EXHIBIT B

IN THE UNITED STATES DISTRICT COURT FOR THE NORTHERN DISTRICT OF OKLAHOMA

STATE OF OKLAHOMA,	)	
	)	
Plaintiff,	)	
	)	
v.	)	**Case No. 05-CV-00329-GKF-SAJ**
	)	
TYSON FOODS, INC., et al.,	)	
	)	
Defendants.	)	

STATE OF OKLAHOMA'S SUPPLEMENTAL RESPONSES TO TYSON FOODS, INC.'S APRIL 3, 2008 REQUESTS FOR PRODUCTION TO THE STATE OF OKLAHOMA

COMES NOW, the Plaintiff, the State of Oklahoma, ex rel. W.A. Drew Edmondson, in his capacity as Attorney General of the State of Oklahoma, and Oklahoma Secretary of the Environment, C. Miles Tolbert, in his capacity as the Trustee for Natural Resources for the State of Oklahoma under CERCLA, (hereinafter "the State") and hereby supplements its response to Tyson Foods, Inc.'s, April 3, 2008 Request for Production. The State reserves the right to supplement these responses. The State hereby incorporates its original General Objections as if fully stated herein.

SUPPLEMENTAL RESPONSE

REQUEST FOR PROUDUCTION NO. 2: Please produce all correspondence between Plaintiffs, Plaintiffs' Experts, Plaintiffs' Attorneys, or any person or agent acting on Plaintiffs' behalf and any publication, association, journal, or other entity regarding the submission for peer review and/or publication as an article, poster, abstract, or in any format of the scientific opinions provided or to be provided by Dr. Valerie J. Harwood in this Lawsuit, including but not limited to Dr. Harwood's development or identification of a "poultry litter marker," Harwood supplemental Aff.¶¶ 2-3.

RESPONSE TO REQUEST NO.2: The State hereby incorporates its previous response and objections to this request as if fully stated herein. Subject to and without waiver of any objection, the following documents, produced on or before May 14, 2008 are responsive to this request:

PI-Harwood 00003206
HarwoodCORR000007
HarwoodCORR000027
HarwoodCORR000028
HarwoodCORR000029
HarwoodCORR000030
HarwoodCORR000031
HarwoodCORR000067
HarwoodCORR000070
HarwoodCORR000071
HarwoodCORR000072
HarwoodCORR000073

In addition, the State is contemporaneously providing HarwoodCORR00000085.

REQUEST FOR PRODUCTION NO. 3: Please produce all correspondence between Plaintiffs, Plaintiffs' Experts, Plaintiffs' Attorneys, or any person or agent acting on Plaintiffs' behalf and any publication, association, journal, or other entity regarding the submission for peer review and/or publication as an article, poster, abstract, or in any format of the scientific opinions provided or to be provided by Dr. Roger Olsen in this Lawsuit, including but not limited to Dr. Olsen's development or identification of a "definitive poultry waste signature," Olsen Aff.¶ 6.

RESPONSE TO REQUEST NO.3: The State hereby incorporates its previous response and objections to this request as if fully stated herein. Subject to and without waiver of any objection, the following documents, produced on or about May 14, 2008 are responsive to this request:

OlsenCORR0015605
OlsenCORR0015757
OlsenCORR0015758
OlsenCORR0015760
OlsenCORR0015774
OlsenCORR0015775
OlsenCORR0015758
OlsenCORR0015759
OlsenCORR0015779
OlsenCORR0015781
OlsenCORR0015782
OlsenCORR0015783
OlsenCORR0015784
OlsenCORR0015790
OlsenCORR0015795
OlsenCORR0016070
OlsenCORR0016074
OlsenCORR0016297
OlsenCORR0016298
OlsenCORR0016299
OlsenCORR0016308
OlsenCORR0016312
OlsenCORR0016332
OlsenCORR0016996
OlsenCORR0016997
OlsenCORR0016998
OlsenCORR0017644
OlsenCORR0017648
OlsenCORR0017649
OlsenCORR0017653
OlsenCORR0017654
OlsenCORR0017659
OlsenCORR0017660
OlsenCORR0017661

OlsenCORR0017670
OlsenCORR0017672
OlsenCORR0017674
OlsenCORR0017676
OlsenCORR0017678
OlsenCORR0017679
OlsenCORR0017680
OlsenCORR0017681
OlsenCORR0017682
OlsenCORR0017683
OlsenCORR0017684
OlsenCORR0017685
OlsenCORR0017687
OlsenCORR0017688
OlsenCORR0017689
OlsenCORR0017692
OlsenCORR0017693
OlsenCORR0019274
OlsenCORR0019281
OlsenCORR0019290
OlsenCORR0019292
OlsenCORR0019293
OlsenCORR0019322
OlsenCORR0019324
OlsenCORR0019326
OlsenCORR0019327
OlsenCORR0019330
OlsenCORR0019333
OlsenCORR0019334
OlsenCORR0019735
OlsenCORR0019748
OlsenCORR0019750
OlsenCORR0019751

REQUEST FOR PRODUCTION NO.5: Please produce all materials, including but not limited to any drafts or versions of any article, poster, abstract, or material in any other format, with all supporting data, figures, tables, illustrations, references, and appendices, submitted or made available to any publication, association, journal, or other entity for peer review and/or publication regarding the scientific opinions provided or to be provided by Dr. Valerie J.

Harwood in this Lawsuit, including but not limited to Dr. Harwood's development or identification of a "poultry litter marker," Harwood Supplemental Aff. ¶¶ 2-3.

RESPONSE TO REQUEST NO.5: The State hereby incorporates its previous response and objections to this request as if fully stated herein. Subject to and without waiver of any objection, the State refers Defendants to Harwood 00000092_PoultryLitterPCR_MS_FINAL_2_.pdf and Harwood00000093 AEMTMP-02130-08_1 and Harwood 00000094 .pdf, which are attached hereto.

REQUEST FOR PRODUCTION NO. 6: Please produce all materials, including but not limited to any drafts or versions of any article, poster, abstract, or material in any other format, with all supporting data, figures, tables, illustrations, references, and appendices, submitted or made available to any publication, association, journal, or other entity for peer review and/or publication regarding the scientific opinions provided or to be provided by Dr. Roger Olsen in this Lawsuit, including but not limited to Dr. Olsen's development or identification of a "definitive poultry waste signature," Olsen Aff. ¶ 6.

RESPONSE TO REQUEST NO.6: The State hereby incorporates its previous response and objections to this request as if fully stated herein. Subject to and without waiver of any objection, see documents referenced in response to request no. 3. Additionally, the State is not aware of any materials submitted for peer review responsive to this request. The State will supplement its response to this request if additional information becomes available.

Respectfully Submitted,

W.A. Drew Edmondson OBA # 2628
ATTORNEY GENERAL
Kelly H. Burch OBA #17067
J. Trevor Hammons OBA #20234
Daniel P. Lennington OBA #21577
ASSISTANT ATTORNEYS GENERAL
State of Oklahoma
313 N.E. 21st St.
Oklahoma City, OK 73105
(405) 521-3921

Robert A. Nance

M. David Riggs OBA #7583
Joseph P. Lennart OBA #5371
Richard T. Garren OBA #3253
Sharon K. Weaver OBA #19010
Robert A. Nance OBA #6581
D. Sharon Gentry OBA #15641
David P. Page OBA #6852
RIGGS, ABNEY, NEAL, TURPEN,
ORBISON & LEWIS
502 West Sixth Street
Tulsa, OK 74119
(918) 587-3161

Louis W. Bullock OBA #1305
Robert M. Blakemore OBA 18656
BULLOCK, BULLOCK & BLAKEMORE
110 West Seventh Street Suite 707
Tulsa OK 74119
(918) 584-2001

Frederick C. Baker
(admitted *pro hac vice*)
Lee M. Heath
(admitted *pro hac vice)*
Elizabeth C. Ward
(admitted *pro hac vice*)
Elizabeth Claire Xidis
(admitted *pro hac vice*)
MOTLEY RICE, LLC
28 Bridgeside Boulevard
Mount Pleasant, SC 29465
(843) 216-9280

William H. Narwold
(admitted *pro hac vice*)
Ingrid L. Moll
(admitted *pro hac vice)*
MOTLEY RICE, LLC
20 Church Street, 17th Floor
Hartford, CT 06103
(860) 882-1676

Jonathan D. Orent
(admitted *pro hac vice)*
Michael G. Rousseau
(admitted *pro hac vice)*
Fidelma L. Fitzpatrick
(admitted *pro hac vice)*
MOTLEY RICE, LLC
321 South Main Street
Providence, RI 02940
(401) 457-7700

Attorneys for the State of Oklahoma

CERTIFICATE OF SERVICE

I hereby certify that on this 16th day of July, 2008, I electronically transmitted the above and foregoing pleading to the Clerk of the Court using the ECF System for filing and a transmittal of a Notice of Electronic Filing to the following ECF registrants:

W. A. Drew Edmondson, Attorney General	fc_docket@oag.state.ok.us
Kelly H. Burch, Assistant Attorney General	kelly_burch@oag.state.ok.us
J. Trevor Hammons, Assistant Attorney General	trevor_hammons@oag.state.ok.us
Daniel P. Lennington, Assistant Attorney General	daniel.lennington@oag.ok.gov
M. David Riggs	driggs@riggsabney.com
Joseph P. Lennart	jlennart@riggsabney.com
Richard T. Garren	rgarren@riggsabney.com
Douglas A. Wilson	doug_wilson@riggsabney.com
Sharon K. Weaver	sweaver@riggsabney.com
Robert A. Nance	rnance@riggsabney.com
D. Sharon Gentry	sgentry@riggsabney.com
David P. Page	dpage@riggsabney.com
RIGGS, ABNEY, NEAL, TURPEN, ORBISON & LEWIS	
Louis Werner Bullock	lbullock@bullock-blakemore.com
Robert M. Blakemore	bblakemore@bullock-blakemore.com
BULLOCK, BULLOCK & BLAKEMORE	

Frederick C. Baker	fbaker@motleyrice.com
Lee M. Heath	lheath@motleyrice.com
Elizabeth C. Ward	lward@motleyrice.com
Elizabeth Claire Xidis	cxidis@motleyrice.com
William H. Narwold	bnarwold@motleyrice.com
Ingrid L. Moll	imoll@motleyrice.com
Jonathan D. Orent	jorent@motleyrice.com
Michael G. Rousseau	mrousseau@motleyrice.com
Fidelma L. Fitzpatrick	ffitzpatrick@motleyrice.com

MOTLEY RICE, LLC
Counsel for State of Oklahoma

Robert P. Redemann	rredemann@pmrlaw.net
Lawrence W. Zeringue	lzeringue@pmrlaw.net
David C. Senger	dsenger@pmrlaw.net

PERRINE, MCGIVERN, REDEMANN, REID, BARRY & TAYLOR, P.L.L.C.

Robert E Sanders	rsanders@youngwilliams.com
Edwin Stephen Williams	steve.williams@youngwilliams.com

YOUNG WILLIAMS P.A.
Counsel for Cal-Maine Farms, Inc and Cal-Maine Foods, Inc.

John H. Tucker	jtucker@rhodesokla.com
Theresa Noble Hill	thill@rhodesokla.com
Colin Hampton Tucker	ctucker@rhodesokla.com
Leslie Jane Southerland	ljsoutherland@rhodesokla.com

RHODES, HIERONYMUS, JONES, TUCKER & GABLE

Terry Wayen West	terry@thewestlawfirm.com

THE WEST LAW FIRM

Delmar R. Ehrich	dehrich@faegre.com
Bruce Jones	bjones@faegre.com
Krisann C. Kleibacker Lee	kklee@faegre.com
Todd P. Walker	twalker@faegre.com

FAEGRE & BENSON, LLP

Dara D. Mann	dmann@mckennalong.com

MCKENNA, LONG & ALDRIDGE, LLP
Counsel for Cargill, Inc. & Cargill Turkey Production, LLC

James Martin Graves	jgraves@bassettlawfirm.com
Gary V Weeks	gweeks@bassettlawfirm.com

Paul E. Thompson, Jr	pthompson@bassettlawfirm.com
Woody Bassett	wbassett@bassettlawfirm.com
Jennifer E. Lloyd	jlloyd@bassettlawfirm.com
BASSETT LAW FIRM	
George W. Owens	gwo@owenslawfirmpc.com
Randall E. Rose	rer@owenslawfirmpc.com
OWENS LAW FIRM, P.C.	

Counsel for George's Inc. & George's Farms, Inc.

A. Scott McDaniel	smcdaniel@mhla-law.com
Nicole Longwell	nlongwell@mhla-law.com
Philip Hixon	phixon@mhla-law.com
Craig A. Merkes	cmerkes@mhla-law.com
MCDANIEL, HIXON, LONGWELL & ACORD, PLLC	
Sherry P. Bartley	sbartley@mwsgw.com
MITCHELL, WILLIAMS, SELIG, GATES & WOODYARD, PLLC	

Counsel for Peterson Farms, Inc.

John Elrod	jelrod@cwlaw.com
Vicki Bronson	vbronson@cwlaw.com
P. Joshua Wisley	jwisley@cwlaw.com
Bruce W. Freeman	bfreeman@cwlaw.com
D. Richard Funk	rfunk@cwlaw.com
CONNER & WINTERS, LLP	

Counsel for Simmons Foods, Inc.

Stephen L. Jantzen	sjantzen@ryanwhaley.com
Paula M. Buchwald	pbuchwald@ryanwhaley.com
Patrick M. Ryan	pryan@ryanwhaley.com
RYAN, WHALEY, COLDIRON & SHANDY, P.C.	
Mark D. Hopson	mhopson@sidley.com
Jay Thomas Jorgensen	jjorgensen@sidley.com
Timothy K. Webster	twebster@sidley.com
Thomas C. Green	tcgreen@sidley.com
Gordon D. Todd	gtodd@sidley.com
SIDLEY, AUSTIN, BROWN & WOOD LLP	
Robert W. George	robert.george@tyson.com
L. Bryan Burns	bryan.burns@tyson.com
TYSON FOODS, INC	

Michael R. Bond	michael.bond@kutakrock.com
Erin W. Thompson	erin.thompson@kutakrock.com
KUTAK ROCK, LLP	

Counsel for Tyson Foods, Inc., Tyson Poultry, Inc., Tyson Chicken, Inc., & Cobb-Vantress, Inc.

R. Thomas Lay	rtl@kiralaw.com
KERR, IRVINE, RHODES & ABLES	
Jennifer Stockton Griffin	jgriffin@lathropgage.com
David Gregory Brown	
LATHROP & GAGE LC	

Counsel for Willow Brook Foods, Inc.

Robin S Conrad	rconrad@uschamber.com
NATIONAL CHAMBER LITIGATION CENTER	
Gary S Chilton	gchilton@hcdattorneys.com
HOLLADAY, CHILTON AND DEGIUSTI, PLLC	

Counsel for US Chamber of Commerce and American Tort Reform Association

D. Kenyon Williams, Jr.	kwilliams@hallestill.com
Michael D. Graves	mgraves@hallestill.com
HALL, ESTILL, HARDWICK, GABLE, GOLDEN & NELSON	

Counsel for Poultry Growers/Interested Parties/ Poultry Partners, Inc.

Richard Ford	richard.ford@crowedunlevy.com
LeAnne Burnett	leanne.burnett@crowedunlevy.com
CROWE & DUNLEVY	

Counsel for Oklahoma Farm Bureau, Inc.

Kendra Akin Jones, Assistant Attorney General	Kendra.Jones@arkansasag.gov
Charles L. Moulton, Sr Assistant Attorney General	Charles.Moulton@arkansasag.gov

Counsel for State of Arkansas and Arkansas National Resources Commission

Mark Richard Mullins	richard.mullins@mcafeetaft.com
MCAFEE & TAFT	

Counsel for Texas Farm Bureau; Texas Cattle Feeders Association; Texas Pork Producers Association and Texas Association of Dairymen

Mia Vahlberg	mvahlberg@gablelaw.com
GABLE GOTWALS	

James T. Banks	jtbanks@hhlaw.com
Adam J. Siegel	ajsiegel@hhlaw.com
HOGAN & HARTSON, LLP	

Counsel for National Chicken Council; U.S. Poultry and Egg Association & National Turkey Federation

John D. Russell	jrussell@fellerssnider.com
FELLERS, SNIDER, BLANKENSHIP, BAILEY & TIPPENS, PC	

William A. Waddell, Jr.	waddell@fec.net
David E. Choate	dchoate@fec.net
FRIDAY, ELDREDGE & CLARK, LLP	

Counsel for Arkansas Farm Bureau Federation

Barry Greg Reynolds	reynolds@titushillis.com
Jessica E. Rainey	jrainey@titushillis.com
TITUS, HILLIS, REYNOLDS, LOVE, DICKMAN & MCCALMON	

Nikaa Baugh Jordan	njordan@lightfootlaw.com
William S. Cox, III	wcox@lightfootlaw.com
LIGHTFOOT, FRANKLIN & WHITE, LLC	

Counsel for American Farm Bureau and National Cattlemen's Beef Association

Also on this 16th day of July, 2008, I mailed a copy of the above and foregoing pleading to the following:

David Gregory Brown
Lathrop & Gage, LC
314 E. High Street
Jefferson City, MO 65101

Thomas C. Green
Sidley Austin Brown & Wood, LLP
1501 K St. NW
Washington, DC 20005

Cary Silverman
Victor E. Schwartz
Shook Hardy & Bacon LLP
600 14th St. NW, Ste. 800
Washington, DC 20005-2004

C. Miles Tolbert
Secretary of the Environment
State of Oklahoma
3800 North Classen
Oklahoma City, OK 73118

Dustin McDaniel
Justin Allen
Office of the Attorney General (Little Rock)
323 Center Street, Suite 200
Little Rock, AR 72201-2610

Steven B. Randall
58185 County Road 658
Kansas, Ok 74347

George R. Stubblefield
HC 66, Box 19-12
Proctor, Ok 74457

Robert A. Nance
Robert A. Nance

Ward, Liza

From: Harwood, Valerie [vharwood@cas.usf.edu]
Sent: Thursday, July 10, 2008 4:07 PM
To: Ward, Liza; David Page
Subject: FW: Manuscript submission (AEM01306-08 Version 1)

Attachments: PoultyLitterQPCR_MS_FINAL.doc; AEMTMP-02130-08_1[1].pdf

PoultyLitterQPCRAEMTMP-02130-
_MS_FINAL.doc .3_1[1].pdf (140 .

Email forwarded as requested

Valerie J.(Jody) Harwood, Ph.D.
Department of Biology, SCA 110
University of South Florida
4202 E. Fowler Ave.
Tampa, FL 33620
(813) 974-1524 - phone
(813) 974-3263 - fax

-----Original Message-----
From: Harwood, Valerie
Sent: Wednesday, June 11, 2008 3:37 PM
To: Jennifer Weidhaas (jweidhaas@northwind-inc.com); Tamzen MacBeth (tmacbeth@northwind-inc.com); Olsen Roger (olsenrl@cdm.com); David Page(new)
Subject: FW: Manuscript submission (AEM01306-08 Version 1)

Manuscript submitted!!

Valerie J.(Jody) Harwood, Ph.D.
Department of Biology, SCA 110
University of South Florida
4202 E. Fowler Ave.
Tampa, FL 33620
(813) 974-1524 - phone
(813) 974-3263 - fax

-----Original Message-----
From: journalsrr@asmusa.org [mailto:journalsrr@asmusa.org]
Sent: Wednesday, June 11, 2008 3:33 PM
To: Harwood, Valerie
Subject: Manuscript submission (AEM01306-08 Version 1)

Dr. Valerie Harwood
University of South Florida
Dept. of Biology
4202 East Fowler Ave.
Tampa, FL 33620-5550
United States

Re: Identification and Validation of a Poultry Litter-Specific Biomarker and Development of a 16S rRNA Based Quantitative PCR Assay
(AEM01306-08 Version 1)

Dear Dr. Harwood:

You have successfully submitted your manuscript via the Rapid Review system. The control number of your manuscript is AEM01306-08 Version 1.
Take note of this number, and refer to it in any correspondence with the Journals Department or with the editor. You may log onto the Rapid Review system at any time to see the current status of your manuscript and the name of the editor handling it. The URL is http://www.rapidreview.com/ASM2/author.html, and your user name is vharwood. To find contact information for the editor handling your manuscript, go to the following URL: http://www.asm.org/journals/editors.asp

In submitting your manuscript to Applied and Environmental Microbiology (AEM), the author(s) guarantees that a manuscript with substantially the same content has not been submitted or published elsewhere and that all of the authors are aware of and agree to the submission.

By publishing in the journal, the authors agree that any DNAs, viruses, microbial strains, mutant animal strains, cell lines, antibodies, and similar materials newly described in the article are available from a national collection or will be made available in a timely fashion, at reasonable cost, and in limited quantities to members of the scientific community for noncommercial purposes. The authors guarantee that they have the authority to comply with this policy either directly or by means of material transfer agreements through the owner.

Similarly, the authors agree to make available computer programs, originating in the authors' laboratory, that are the only means of confirming the conclusions reported in the article but that are not available commercially. The program(s) and suitable documentation regarding its (their) use may be provided by any of the following means: (i) as a program transmitted via the Internet, (ii) as an Internet server-based tool, or (iii) as a compiled or assembled form on a suitable medium (e.g., magnetic or optical). It is expected that the material will be provided in a timely fashion and at reasonable cost to members of the scientific community for noncommercial purposes. The authors guarantee that they have the authority to comply with this policy either directly or by means of material transfer agreements through the owner.

If your manuscript is accepted for publication, a condition of acceptance is that you assign copyright to the American Society for Microbiology. A copyright transfer agreement is sent with each letter of acceptance after the manuscript has been scheduled for publication.

If your manuscript is accepted for publication in a 2008 issue, page charges (subject to change without notice) will be assessed at $65 per printed page for the first eight pages and $200 for each page in excess of eight for a corresponding author who is an ASM member or $75 per printed page for the first eight pages and $250 for each page in excess of eight for a nonmember corresponding author. A corresponding author who is not a member may join ASM to obtain the member rate. If the research was not supported, you may send a request for a waiver of page charges to the Director, Journals. For more details, including type of articles not charged, see the Instructions to Authors.

IMPORTANT NOTICE: For its primary-research journals, ASM posts online PDF versions of manuscripts that have been peer reviewed and accepted but not yet copyedited. This feature is called "AEM Accepts" and is accessible from the Journals website. The manuscripts are published online as soon as possible after acceptance, on a weekly basis, before the copyedited, typeset versions are published. They are posted "As Is" (i.e., as submitted by the authors at the modification stage), and corrections/changes are NOT accepted. Accordingly, there may be differences between the AEM Accepts version and the final, typeset version. The manuscripts remain listed on the AEM Accepts page until the final, typeset versions are posted, at which point they are removed from the AEM Accepts page and become available only through links from the final, typeset version. They are under subscription access control until 4 months after the typeset versions are posted, when access to all forms becomes free to everyone. Any supplemental material intended, and accepted, for publication is not posted until publication of the final, typeset article.

Thank you for submitting your manuscript for consideration.

Barbara Slinker
Production Editor
Applied and Environmental Microbiology (AEM)

Identification and Validation of a Poultry Litter-Specific Biomarker and Development of a 16S rRNA Based Quantitative PCR Assay

Jennifer L. Weidhaas[1], Tamzen W. Macbeth[1], Roger L. Olsen[2], Valerie J. Harwood[3, *]

1. North Wind, Inc. 1425 Higham Street, Idaho Falls, Idaho, 83402

2. CDM, 555 17th St., Suite 1100, Denver, CO 80202

3. * Department of Biology, University of South Florida, 4202 E. Fowler Ave., Tampa, Florida 33620, Phone: 813-974-1524, Fax: 813-974-3263 email vharwood@cas.usf.edu

Running title: Brevibacterium marker for fecal source tracking of poulty

ABSTRACT

A poultry litter-specific biomarker was developed for microbial source tracking (MST) in environmental waters. 16S rRNA sequences that were present in fecal-contaminated turkey and chicken litter were identified by terminal restriction fragment length polymorphism (T-RFLP). Cloning and sequencing of potential targets from pools of *E. coli, Bacteroides* or total bacterial DNA yielded four sequences that were ubiquitous in poultry litter and also contained unique sequences for development of target-specific PCR primers. Primer sensitivity and specificity were tested by nested PCR against ten composite poultry litter samples and fecal samples from beef and dairy cattle, swine, ducks, geese, and human sewage. The sequence with greatest sensitivity (100%) and specificity (93.5%) has 98% identity to *Brevibacterium avium,* and was detected in all litter samples. It was detected at low level in only one goose and one duck sample. A quantitative PCR assay was developed and tested on litter, soil and water samples. Litter concentrations were $2.2*10^7$ - $2.5*10^9$ gene copies/g. The biomarker was present in a majority of soil and water samples collected in and near areas where litter was spread, reaching concentrations of 2.9×10^5 gene copies·g^{-1} in soil samples and 5.5×10^7 gene copies·L^{-1} in runoff from the edges of fields. The biomarker will contribute to quantifying the impact of fecal contamination by land-applied poultry litter in this watershed. Furthermore, it has potential for determining fecal source allocations for total maximum daily load (TMDL) programs and ambient water quality assessment, and may be useful in other geographic regions.

INTRODUCTION

Excessive land application of poultry litter as a waste disposal mechanism has been linked to eutrophication of water bodies (28, 35, 39), the spread of pathogens (15, 19, 21), air and soil pollution with metals (11, 33) and groundwater contamination with nitrate (5). Despite these known effects, land application is still the typically practiced disposal method for poultry litter even though viable and economically favorable alternative disposal practices are available (7, 20).

Identification of the source of fecal pollution contaminating a watershed is of particular interest for protection of water resources and the safety of recreational waters. For example, TMDL assessments require identification of the source of contamination, which is also necessary for remediation of impaired waters(44). Current methods for detecting the presence of fecal pollution, which carries an increased risk of the presence of pathogenic microorganisms, involve the cultivation of fecal indicator organisms such as fecal coliforms in the family *Enterobacteriaceae* (Oklahoma Administrative Code, Title 785, Chapter 46). The U.S. EPA and many states recognize *Escherichia coli* and enterococci as indicators of freshwater recreational water quality (42).

Drawbacks to the use of indicator organisms which limit the ability of researchers to pinpoint sources of fecal contamination include the non-specificity of the fecal coliforms to one source (25, 43), variable survival rates of various indicator organisms (1) and the growth or extended persistence of these indicator organisms after release to the environment (12, 45). These drawbacks have lead to research into alternative methods for the assessment of human health risk

from microbial pathogens in recreational waters that do not include the culturing of fecal indicator organisms for identification and quantification of the source of fecal pollution (46).

A variety of microbial source tracking (MST) methods (for recent reviews see (17, 40, 47)) have been proposed as an alternative to cultivation of fecal coliforms. Some of these genotypic molecular based techniques have included library dependent methods (i.e., culture and isolate-based) such as ribotyping (10, 31) and repetitive element polymerase chain reaction (REP-PCR) (14). Library independent methods (i.e., detection of a genetic biomarker in extracted DNA) have also been developed using discovery techniques such as suspension arrays (8), subtractive hybridization (13, 26), and terminal restriction fragment length polymorphism (T-RFLP) (3), among others. Host marker specific targets have included *Enterococcus faecium* (37), *Bifidobacterium* and members of the *Bacteroidales* (3, 22, 38), among others. Relatively few microbial targets specific to poultry fecal material have been identified. To date *Enterococcus faecalis* (23), *E. coli* (10) and *Bacteriodes* (26) have been associated with poultry fecal material, but only the *Bacteroides* biomarker (26) was specifically associated with poultry and not other fecal sources The objective of this research was to identify a poultry litter-specific biomarker, validate its specificity against other sources of fecal material from within and outside the watershed and develop a 16S rRNA based real-time PCR assay for quantifying the biomarker in environmental samples. This work was carried out as part of ongoing litigation in which the plaintiff is the Oklahoma Attorney General.

METHODS

Sample collection. Litter samples were collected from ten separate facilities (poultry houses), nine chicken and one turkey facility. Litter samples were collected from 18 locations within each

poultry house through the entire depth of the litter. The subsamples (total volume of 4 to 5 gallons) from each house were composited, homogenized and split (riffle splitter) before placement into a sterile whirl pack (approximately 500 mL) and shipped on ice to the laboratory for analysis. Litter application areas in fields (soils) were sampled by collecting 20 subsamples on a predetermined grid pattern across a uniform subarea of one to ten acres in size. The zero to two inch sample from six inch soil cores were composited, disaggregated, sieved to 2 mm, ground, homogenized and split. Vegetation, feathers, and rocks were removed. The split soil samples (500 ml) were transported on ice to the laboratory. Nontarget fecal samples for specificity testing were collected as composites from groups of individuals (Table 3). Samples from beef cattle were collected from ten grazing fields, of which five were within the watershed and five were outside the watershed. Two independent duplicate samples were collected for each field, and each duplicate consisted of feces from ten scats. A total of 200 beef cattle scats were collected and composited into 20 samples. Duck and goose samples were collected in the same fashion, consisting of composites from ten individual scats, and independent duplicates were collected for each area. For ducks, three landing areas inside the watershed and two outside the watershed were sampled, while for geese, two landing areas inside and three landing areas outside the watershed were sampled. A total of 100 scats for duck and geese were collected and composited into 10 samples for duck and 10 samples for geese. Composite samples of fecal slurries were collected from swine facilities, one inside the watershed and one outside (2 duplicate samples) and dairy cattle facilities (one inside the watershed and two outside (2 duplicate samples each) human residential septic cleanout trucks (3 samples) and influent of three separate municipal wastewater treatment plants (3 samples). A total of 20 g of each fecal sample other than litter from each site was collected and was placed in a 20 ml, sterile,

polystyrene tube containing 10 ml of 20% glycerol and shipped on dry ice to the laboratory. All fecal samples were homogenized in the glycerol before DNA extraction. Discrete water samples from larger rivers and lakes were collected using a Van Dorn water sampler or with a churn splitter for discrete or composite samples. Samples from larger rivers were typically composites of 3 samples collected on a transect across the width of the river channel. Samples from smaller rivers were collected using automated samplers. Samples collected during high flow events were composited based on flow volume. Base flow samples were collected as grab samples. River samples were placed into sterile 1-L polystyrene bottles in duplicate and shipped on ice to the laboratory where they were filtered. Runoff samples from the litter application areas (e.g. edge of field runoff samples) were collected during or as soon as possible after rainfall events. Samples were collected either with a passive runoff collector for composite samples or with a dip sampler for discrete samples. Runoff samples were placed into sterile 1-L polystyrene bottles in duplicate and shipped on ice to the laboratory where they were filtered. Groundwater samples were collected directly from existing homeowner's wells or from hydraulically driven shallow probes. Spring samples were collected as grab samples or by using a peristaltic pump. All samples were placed into sterile 1-L polystyrene bottles and shipped onice to the laboratory where they were filtered.

Enumeration of Indicator Bacteria. Indicator bacteria (fecal coliforms, *E. coli* and enterococci) were enumerated according to standard methods using multiple tube fermentation (MTF) and calculation of the most probable number according to according to SM-9221F or SM-9230 (APHA, 2005). MTF tubes containing *E. coli* were identified using broth cultures supplemented with (MUG) (SM-9221F) (2).

Soil, Litter and Fecal Sample DNA Extraction. Genomic DNA was extracted from soil, liter and fecal samples with Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc.) following the manufacturer's instructions. Typically 0.25 g of soil or litter was used in each extraction. DNA was purified by size-exclusion chromatography. Sepharose CL-4B (Sigma-Aldrich) was resuspended in Tris-HCL and sterilized by autoclave at 121 °C for at least 20 minutes. Micro-bio spin columns (Bio-Rad Laboratories) were packed with 1 mL of Sepharose CL-4B through centrifugation. Sepharose columns were then washed twice with Tris-HCl buffer (pH 8) and 50 to 150 µl of sample was added. Purified DNA was concentrated with ethanol precipitation and re-eluted in 100 µL sterile water.

Water Sample DNA Extraction. Within 12 hours of receipt at the laboratory all water samples were filtered through a sterile Supor-200, 0.2 µM filter and frozen at -80°C. Filters were then shattered with sterile glass beads and vortexed vigorously for 15 minutes with sterile, DNase, and RNase free water to remove solids and cells from the filters. The cell suspension was removed from the centrifuge tubes by pipette and placed in a 2 mL bead beating tube from the Bio101 Fast®Spin® DNA extraction kits. The cells were centrifuged at 20,000 x g for 10 minutes, and the supernatant was decanted. Genomic DNA was then extracted using the Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc). The extracted DNA was quantified using a Nanodrop® UV-Vis Spectrophotometer.

T-RFLP Analysis. Extracted genomic DNA and/or cloned DNA was amplified with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM) labeled universal bacterial primers 8F-907R (16, 24), with *E.coli* genus specific primers (Tsen, et al. 1998), and *Bacteroidales* specific primers (Bernhard and Field, 2000). All PCR primers targeted the 16S rRNA gene. Triplicate PCR reactions were generated from each DNA extraction, combined and purified

using QIAquick PCR purification Kits (Qiagen). Approximately 200 ng each of PCR product was digested at 37°C for 6 hours with the *Msp*I restriction enzyme (20µ/µL) (New England BioLabs). Samples were denatured by heating to 95° C for 3 minutes followed by cooling to 4°C. The digested fragments were purified by ethanol precipitation.

Primer Design. Primers were designed using the ABI Primer Express v.2 program (Applied Biosystems, Foster City, CA) and were targeted to variable regions between the potential biomarker sequences and sequences of the top 20 closest related organisms in the GenBank database. The BLAST search (Basic Alignment Search Tool, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was used to check the specificity of each primer.

PCR Assay Conditions. PCR was used to amplify approximately 900 bp of the 16S rRNA genes from *Bacteria* for clone library construction. Each 25 µL PCR reaction included 0.4 mg mL^{-1} molecular-grade bovine serum albumin (BSA) (Sigma Chemicals), 1X PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.5 µM of both the forward (8F) (16) and reverse (907R) (24) primer (Invitrogen), 1U Taq DNA polymerase (Promega), 0.2 mM dNTP (Invitrogen), 1 µL DNA template, and molecular-grade water (Promega). Amplification was performed on a PerkinElmer Model 9600 thermocycler using the following conditions: 94 °C for 5 minutes, 30 cycles of 94 °C (1 minute), 55 °C (45 seconds), and 72 °C (2 minute). A final extension at 72 °C for 7 minutes was performed and the PCR products were held at 4°C. Specificity of the PCR primers to the poultry litter biomarker was evaluated with nested PCR by first amplifying non-target fecal samples by universal bacterial primers 8F, 907R and then amplifying by the potential poultry litter biomarker PCR primers. The nested PCR master mix and thermocycler conditions were similar to the universal PCR with the following exceptions: 1) forward and reverse PCR

primers were specific to the potential poultry biomarker as shown in Table 2, 2) the annealing temperature was 60 °C. Amplification by nested PCR was evaluated by gel electrophoresis.

Clone Libraries. Clone libraries were constructed from the original genomic DNA extracted from the soil and litter samples and amplified with either universal bacterial primers 8F-907R (16, 24), targeting the 16S rRNA genes of *Bacteria* or the *E. coli* genus specific primers V1SF-V3AR (41). The TOPO ® Cloning Reaction methods from Invitrogen ™ were followed for clone library construction. Two clone libraries were constructed (targeting *Bacteria* and *E. coli*) from pooled DNA samples (i.e., 1 µl of genomic DNA extract from each sample was added to the PCR reaction for inclusion into the clones) based on the abundance of the various potential biomarkers as evidenced by the T-RFLP profiles.

qPCR Assay Conditions. Quantitative PCR (qPCR) was used to amplify 530 bp of the 16S rRNA gene from *Brevibacterium spp*. DNA samples were diluted to final concentrations of 3 ng/µL DNA. Each 25µL qPCR reaction included: 1X SYBR Green Master Mix (Roche), 0.5 µM of both the forward (LA35F) and reverse primer (LA35R) (Invitrogen), 5 % DMSO, 5 µL of diluted sample DNA, and molecular-grade water (Promega). Amplification was performed in triplicate on a Biorad Chromo4 thermocylcer using the following conditions: 50 °C for 2 minutes, 95 °C for 15 minutes, 45 cycles of 95 °C (30 seconds), 60 °C (30 seconds), and 72 °C (30 seconds) with a plate read. The 45 cycles was followed by a final extension at 50 °C for 5 minutes. Immediately following the final extension was a melting curve from 70 °C to 90 °C, by 0.1 degree increments, holding for 5 seconds with a plate read. DNA standards ranging from $6*10^{-15}$ to 10^{-21} ng/ul were prepared from serial dilutions of clone plasmid DNA containing the sequence of interest and used to develop the standard curve and method detection limit. Gene copy numbers were calculated from concentrations of positive control standards assuming 9.124

$* 10^{14}$ bp/ul of DNA and one gene copy per genome. Detection limits for the qPCR assay were approximately 2000 plasmid copies in *E. coli*/L water and $7.3 * 10^{4}$ plasmid copies in *E. coli*/gram of soil. Nested qPCR was performed by first amplifying DNA with the universal bacterial 16S rRNA 8F (16) and 907R (24) primers. The production of PCR products was confirmed on a 1.5% agarose gel. The 16S rRNA PCR products were purified with the QIAquick PCR purification kit (QIAGEN) were subjected to qPCR as previously described using the LA35F and LA35R primers for the poultry litter biomarker.

Phylogeny. The phylogeny of the LA35 clone was investigated using the following methods. The clone sequences were assembled and aligned with BioEdit v. 7.0.5.3 and sequences were checked for chimeras with the Ribosomal Database Project II Chimera Check program and Bellerophon. The 16S rRNA sequences of the closest neighbors to the clone sequences were downloaded for inclusion in the phylogenic analysis. Multiple sequence alignments were constructed with Clustal W alignment tool and manually aligned in BioEdit. The bootstraps (1000 resamplings), maximum likelihood and distance matrix analysis (Kimura), and the reconstruction of the phylogenetic trees (FITCH) were performed with the Phylip 3.65 package and in particular the programs SEQBOOT, DNAML, DNADIST, FITCH, CONSENSE, and RETREE. The reconstructed phylogenetic tree was visualized with PhyloDraw V. 0.8 (Graphics Application Lab, Pusan National University).

RESULTS

Identification of potential biomarkers by T-RFLP. A total of 20 T-RFLP profiles were generated from the 5 subsamples of each of the two litter and two soil samples. The T-RFs common among the subsamples and representing more than 1% of the community were selected

for cloning and sequencing (Table 1). A total of 3 *E. coli* T-RFs (i.e., T-RF 496.0, 498.9 and 500.8) and 3 *Bacteria* T-RFs (i.e., T-RF142.9, 147.3 and 158.9) were selected for cloning and sequencing. Clone libraries were constructed from PCR products amplified with *E. coli* specific primers (V1SF-V3AR) (41) or universal bacterial primers (8F-907R) (16, 24). A total of 300 plasmids from the clone libraries were randomly picked. T-RFLP analysis was carried out on each plasmid insert to identify which plasmids contained the T-RFs of potential biomarkers. Inserts containing the T-RFs of interest were sequenced and PCR primers were developed for those sequences containing mismatches as compared to BLAST database results of the top 20 closely related organisms. In all 4 PCR primers for members of 4 genera were developed; a *Brevibacterium spp.*, a *Rhodoplanes spp.*, a *Kineococcus spp.* and a *Pantoea ananatis* strain (Table 2). Two *E. coli* T-RFs were from plasmids that did not contain mismatches between the sequence of interest and the sequences of closely related organisms identified in a BLAST search and therefore were not appropriate biomarkers.

Evaluation of biomarkers against fecal samples. The PCR assays developed for the 4 potential biomarkers of poultry litter were tested for amplification against a variety of nontarget fecal samples from within and outside the watershed (Table 3). Only the *Brevibacterium* clone LA35 appeared to be a potential candidate biomarker for poultry litter in that did not amplify in any fecal samples with the exception of weak amplification in one duck and one goose sample from outside the watershed when analyzed with a nested PCR approach (i.e. PCR with universal bacterial primers and then with the *Brevibacterium* clone LA35 primers). The reconstructed phylogenetic tree of the *Brevibacterium* clone LA35 in relationship to other *Brevibacterium* spp. is presented in Figure 1.

Quantification of the poultry litter biomarker in environmental samples. A SYBR green qPCR protocol was developed and optimized using the LA35F and LA35R primers (Table 2) specific to the *Brevibacterium* clone LA35 poultry litter biomarker. The standard curve of the qPCR assay for the biomarker is presented in Figure 2. The detection limit of the qPCR assay was 6 gene copies/ul of extracted DNA.

Environmental samples from the potential poultry litter impacted watershed were tested for the presence of the biomarker with the qPCR assay (Table 4). A variety of samples from within the watershed were tested, some of which were expected to contain the biomarker (e.g., litter, contaminated soil, runoff samples), some of which had variable potential for higher biomarker levels (e.g., surface water), and some of which had lower potential for biomarker presence (i.e., groundwater samples).

The correlation between the poultry litter biomarker concentration (i.e., as quantified by qPCR) in water and litter samples and *E. coli* and *Enterococcus* as measured by most probable number is presented in Figures 3 and 4. In general the *Enterococcus* MPN counts were well correlated with the concentration of the biomarker in litter ($R^2 = 0.75$) and with the biomarker concentration in water samples ($R^2 = 0.89$). The correlation between *E. coli* concentrations and the biomarker in water samples was also strong ($R^2 = 0.85$) while *E. coli* was less tightly (but significantly) correlated with the biomarker in litter samples ($R^2 = 0.28$). Correlation of the biomarker with *E. coli* and *Enterococcous* spp. provides a line of evidence of the human health risk associated with the runoff from poultry litter application to fields although there is evidence that regrowth of these organisms is possible once they are introduced into the environment (36).

DISCUSSION

The *Brevibacterium* sp. poultry litter biomarker developed in this study was validated in terms of sensitivity (100%) against numerous positive (poultry litter) samples from different locations with the watershed and for specificity (93.5%) against composite non-target fecal samples. These practices are in accordance with recent critical reviews (34, 40) that strongly recommend MST method validation. Future efforts will attempt to extend the method validation outside the watershed and possible outside the region as this biomarker could be useful for identifying fecal pollution sources in other river systems and coastal waters.

The *Brevibacterium* clone LA35 poultry litter biomarker was most closely related to *Brevibacterium avium*, which is associated with bumble-foot lesions in poultry (32). *Brevibacterium* spp. were recently identified in spent mushroom compost that was originally derived from chicken litter and cereal straw (29). Additionally *Brevibacterium avium, Brevibacterium iodinum,* and *Brevibacterium epidermidis* were found to represent more than 7% of a 16S rRNA clone library originating from broiler chicken litter (27). Certain *Brevibacterium* spp. are associated with milk and cheese curds(6), human skin(9), and soils (30). *Brevibacterium* spp. have been associated with disease in humans although to date these opportunistic pathogens have only been isolated from immunocompromised patients (4, 9, 18).

As poultry litter is land-applied as a disposal practice (19, 33, 35), it was important to identify a marker that could survive the process of deposition on bedding and spreading on fields. Therefore, the T-RFLP screening process included both litter and contaminated soil samples. This strategy allowed for the rapid elimination of numerous targets that could be abundant in the poultry fecal material, but not as abundant in the litter and not present in the environment after

litter application. This strategy for marker identification is in contrast with the work by Lu and colleagues (2007) where a genome fragment enrichment method was used to identify microbial sequences specific to chicken feces. Based on the PCR assays developed from clone libraries of the genome fragments, 6 to 40% of the chicken fecal samples collected from a wide geographic region contained DNA that could be amplified by the various assays (26). In comparison the LA35 biomarker was found in all the poultry litter samples tested, although it should be noted that all of the samples were collected in the Oklahoma/Arkansas region.

The examination of environmental samples from within the poultry litter impacted watershed suggest a correlation between the application of poultry litter to a field and concentration of the biomarker in the receiving waters, as evidenced by the generally decreasing trend in biomarker concentration with decreasing concentration of fecal indicator organisms. These results indicate that the watershed is in fact being impacted by the application of poultry litter to fields within the watershed. However, the magnitude of the impact as measured by the distribution of the biomarker within the watershed cannot be quantified with the limited number of environmental samples processed to date. Future work will include the testing of environmental samples from within the watershed by the qPCR assay to evaluate the distribution of the poultry litter-specific biomarker as compared to indicator bacteria, antibiotics and heavy metals. Additionally, testing of the poultry litter-specific biomarker against more fecal samples from other watersheds and additional avian fecal material will be conducted as the LA35 poultry litter biomarker was found in low abundance (i.e., a nested PCR approach was required for detection) in two non-target composite avian fecal samples (i.e., a duck and a goose sample) from outside the watershed.

Conclusions

In summary a novel biomarker of poultry litter was identified and a 16S rRNA based real-time PCR assay was developed for this biomarker. The specificity of the assay (93.5%) was tested against 31 separate non-target fecal samples and sensitivity was tested against10 target litter samples (100%). The field applicability of the assay was evaluated by testing for the biomarker in environmental samples expected to have variable concentrations of the biomarker, which we hypothesized would be correlated with the concentration of fecal indicator bacteria. A generally positive correlation was found between biomarker concentration and fecal indicator bacteria concentration which was particularly strong for enterococci. The research presented herein is the first identification of a *Brevibacterium* spp. for microbial source tracking studies and is among the first quantifiable method for tracking of poultry fecal sources in environmental waters.

ACKNOWLEDGMENTS

This research was conducted in connection with work performed as retained experts in a pending legal case brought by the State of Oklahoma against several poultry integrators. Drs. Harwood & Olsen have been retained to serve as expert witnesses by the State of Oklahoma and have provided testimony regarding this research.

The authors are grateful for the assistance provided by Kyle Collins, William Blackmore, James Jackson, Erin O'Leary Jeapson and Michelle Andrews. Additionally the authors acknowledge the Molecular Research Core Facility at Idaho State University for graciously allowing us the use of their laboratory space and equipment.

References

1. **Anderson, K., J. Whitlock, and V. Harwood.** 2005. Persistence and Differential Survival of Fecal Indicator Bacteria in Subtropical Waters and Sediments. Applied and Environmental Microbiology **71:**3041-3048.

2. **APHA.** 2005. Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association, Inc., Washington, D.C.

3. **Bernhard, A., and K. Field.** 2000. Identification of Nonpoint Sources of Fecal Pollution in Coastal Waters by Using Host-Specific 16S Ribosomal DNA Genetic Markers from Fecal Anaerobes. Applied and Environmental Microbiology **66:**1587-1594.

4. **Beukinga, I., H. Rodriguez-Villalobos, A. Deplano, F. Jacobs, and M. Struelens.** 2005. Management of long-term catheter-related *Brevibacterium* bacteraemia. Clinical Microbiology and Infection **10:**465-467.

5. **Bitzer, C., and J. Sims.** 1988. Estimating the availability of nitrogen in poultry manure through laboratory and field studies. Journal of Environmental Quality **17:**47-54.

6. **Brennan, N., A. Ward, T. Beresford, P. Fox, M. Goodfellow, and T. Cogan.** 2002. Biodiversity of the bacterial flora on the surface of a smear cheese. Applied and Environmental Microbiology **68:**820-830.

7. **Bujozcek, G., J. Oleszkiewicz, R. Sparling, and S. Cenkowski.** 2000. High Solid Anaerobic Digestion of Chicken Manure. Journal of Agricultural Engineering Research **76:**51-60.

8. **Call, D., D. Satterwhite, and M. Soule.** 2007. Using DNA suspension arrays to identify library-independent markers for bacterial source tracking. Applied and Environmental Microbiology **41:**3740-3746.

9. **Cannon, J., F. Spadoni, S. Pesh-Iman, and S. Johnson.** 2005. Pericardial infection caused by *Brevibacterium casei*. Clinical Microbiology and Infection **11:**164-165.

10. **Carson, C., B. Shear, M. Ellersieck, and A. Asfaw.** 2001. Identification of Fecal *Escherichia coli* from Humans and Animals by Ribotyping. Applied and Environmental Microbiology **67:**1503-1507.

11. **Connor, R., M. Connor, K. Irgolic, J. Sabrsula, H. Gurleyuk, R. Brunette, C. Howard, J. Garcia, J. Brien, J. Brien, and J. Brien.** 2005. Transformations, Air Transport and Human Impact of Arsenic from Poultry Litter. Environmental Forensics **6:**83-89.

12. **Desmarais, T., H. Solo-Gabriele, and C. Palmer.** 2002. Influence of Soil on Fecal Indicator Organisms in a Tidally Influenced Subtropical Environment. Applied and Environmental Microbiology **68:**1165-1172.

13. **Dick, L. K., A. E. Bernhard, T. J. Brodeur, J. W. Santo Domingo, J. M. Simpson, S. P. Walters, and K. G. Field.** 2005. Host distributions of uncultivated fecal *Bacteroidales* bacteria reveal genetic markers for fecal source identification. Applied and Environmental Microbiology **71:**3184-3191.

14. **Dombek, P., L. Johnson, S. Zimmerley, and M. Sadowsky.** 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. Applied and Environmental Microbiology **66:**2572-2577.

15. **Edwards, D., and T. Daniel.** 1994. A comparison of runoff quality effects of organic and inorganic fertilizers applied to fescue grass plots. Journal of the American Water Resources Association **30:**35-41.

16. **Edwards, U., T. Rogall, H. Blocker, M. Emde, and E. Bottger.** 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Research **17:**7843-7853.

17. **Field, K., and M. Samadpour.** 2007. Fecal source tracking, the indicator paradigm, and managing water quality. Water Research **41:**3517-3538.

18. **Janda, W., P. Tipirneni, and R. Novak.** 2003. *Brevibacterium casei* Bacteremia and Line Sepsis in a Patient with AIDS. Journal of Infection **46:**61-64.

19. **Jenkins, M., D. Endale, H. Schomber, and R. Sharpe.** 2006. Fecal bacteria and sex hormones in soil and runoff from cropped watersheds amended with poultry litter. Science of the Total Environment **358:**164-177.

20. **Kelleher, B., J. Leahy, A. Henihan, T. O'Dwyer, D. Sutton, and M. Leahy.** 2002. Advances in poultry litter disposal technology – a review. Bioresource Technology **83:**27-36.

21. **Kelley, T., O. Pancorbo, W. Mercka, S. Thompson, M. Cabrera, and H. Barnhart.** 1994. Fate of Selected Bacterial Pathogens and Indicators in Fractionated Poultry Litter During Storage. Journal of Applied Poultry Research **3:**279-288.

22. **Kildare, B. J., C. M. Leutenegger, B. S. McSwain, D. G. Bambic, V. B. Rajal, and S. Wuertz.** 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. Water Research **41:**3701-3715.

23. **Kuntz, R., P. Hartel, J. Rodgers, and W. Segars.** 2004. Presence of *Enterococcus faecalis* in broiler litter and wild bird feces for bacterial source tracking. Water Research **38:**3551-3557.

24. **Lane, D.** 1991. 16S/23S rRNA sequencing. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid sequencing techniques in bacterial systematics. John Wiley and Sons, New York, N.Y.

25. **Leclerc, H., D. Mossel, S. Edberg, and C. Struijk.** 2001. Advances in the Bacteriology of the Coliform Group: Their Suitability as Markers of Microbial Water Safety. Annual Reviews in Microbiology **55:**201-234.

26. **Lu, J., J. Domingo, and O. Shanks.** 2007. Identification of a chicken-specific fecal microbial sequences using a metagenomic approach. Water Research **41:**3561-3574.

27. **Lu, J., S. Sanchez, C. Hofacre, J. Maurer, B. Harmon, and M. Lee.** 2003. Evaluation of Broiler Litter with Reference to the Microbial Composition as Assessed by Using 16S rRNA and Functional Gene Markers. Applied and Environmental Microbiology **69:**901-908.

28. **Mozaffari, M., and J. Sims.** 1994. Phosphorus availability and sorption in an Atlantic coastal plain watershed dominated by animal-based agriculture. Soil Science **157:**97-107.

29. **Ntougias, S., G. Zervakis, N. Kavroulakis, C. Ehaliotis, and K. Papadopoulou.** 2004. Bacterial Diversity in Spent Mushroom Compost Assessed by Amplified rDNA Restriction Analysis and Sequencing of Cultivated Isolates. Systematic and Applied Microbiology **27:**746-754.

30. **Onraedt, A., W. Soetaert, and E. Vandamme.** 2005. Industrial importance of the genus *Brevibacterium*. Biotechnology Letters **27:**527-533.

31. **Parveen, S., R. Murphree, L. Edmiston, C. Kaspar, and M. Tamplin.** 1999. Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human

and nonhuman sources of fecal pollution. Applied and Environmental Microbiology **65:**3142-3147.

32. **Pascual, C., and M. Collins.** 1999. *Brevibacterium avium* sp. nov., isolated from poultry. International Journal of Systematic Bacteriology **49:**1527-1530.

33. **Pirani, A., K. Brye, T. Daniel, B. Haggard, E. Gbur, and J. Mattice.** 2006. Soluble Metal Leaching from a Poultry Litter–Amended Udult under Pasture Vegetation. Vadose Zone Journal **5:**1017-1034.

34. **Santo Domingo, J., D. Bambic, T. Edge, and S. Wuertz.** 2007. Quo vadis source tracking? Towards a strategic framework for environmental monitoring of fecal pollution. Water Research **41:**3539-3552.

35. **Schroeder, P., D. Radcliffer, and M. Cabrera.** 2004. Rainfall Timing and Poultry Litter Application Rate Effects on Phosphorus Loss in Surface Runoff. Journal of Environmental Quality **33:**2201-2209.

36. **Scott, T., J. Rose, T. Jenkins, S. Farrah, and J. Lukasik.** 2002. Microbial Source Tracking: Current Methodology and Future Directions. Applied and Environmental Microbiology **68:**5796-5803.

37. **Scott, T. M., T. M. Jenkins, J. Lukasik, and J. B. Rose.** 2005. Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. Environmental Science and Technology **39:**283-287.

38. **Seurinck, S., T. Defoirdt, W. Verstraete, and S. D. Siciliano.** 2005. Detection and quantification of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. Environmental Microbiology **7:**249-259.

39. **Sharpley, A., T. Daniel, J. Sims, and D. Pote.** 1996. Determining environmentally sound soil phosphorus levels. Journal of Soil and Water Conservation **51:**160-168.

40. **Stoeckel, D., and V. Harwood.** 2007. Performance, design and analysis in microbial source tracking studies. Applied and Environmental Microbiology **73:**2405-2415.

41. **Tsen, H., C. Lin, and W. Chi.** 1998. Development and use of 16S rRNA gene targeted PCR primers for the identification of *Escherichia coli* cells in water. Journal of Applied Microbiology **85:**554-560.

42. **USEPA.** 2000. Improved enumeration methods for the recreational water quality indicators: enterococci and *Escherichia coli.* EPA-821/R-771 97/004. U.S. Environmental Protection Agency.

43. **USEPA.** 2005. Microbial source tracking guide document, EPA/600/R-05/064. U.S. Environmental Protection Agency.

44. **USEPA.** 2001. Protocol for developing pathogen TMDLs. EPA 841-R-00-002. U.S. Environmental Protection Agency.

45. **Van Donsel, D., E. Geldreich, and N. Clarke.** 1967. Seasonal Variations in Survival of Indicator Bacteria in Soil and their Contribution to Storm-water Pollution. Applied Microbiology **15:**1362-1370.

46. **Wade, T., R. Calderon, E. Sams, M. Beach, K. Brenner, A. Williams, and A. Dufour.** 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspectives **114:**24-28.

47. **Wuertz, S., and J. Field.** 2007. Emerging microbial and chemical source tracking techniques to identify origins of fecal contamination in waterways. Water Research **41:**3515-3516.

Table 1. Common T-RFs among replicates from two fecal-contaminated poulty litter samples and two soils to which the litter had been applied.

	Number of subsamples tested (number containing T-RF of interest)			
T-RF	Litter A	Litter B	Soil A	Soil B
E.coli PCR products, digested with *MspI*				
496.0	4 (4)	5 (4)	5 (3)	5 (5)
498.9	4 (4)	5 (5)	5 (4)	5 (5)
500.8	4 (4)	5 (5)	5 (5)	5 (5)
Universal bacteria PCR products, digested with *MspI*				
80.1	4 (4)	5 (5)	5 (0)	3 (3)
130.9	4 (3)	5 (5)	5 (1)	3 (0)
142.9	4 (4)	5 (4)	5 (2)	3 (2)
147.3	4 (4)	5 (5)	5 (5)	3 (2)
158.9	4 (3)	5 (5)	5 (4)	3 (2)
165.0	4 (3)	5 (5)	5 (4)	3 (2)

*Underlined T-RFs correlate to those organisms for which PCR primers were developed

Table 2. Nucleotide sequences and targets of primers used in this study.

Primer	Target	Sequence (5'-3')	Position	Tm (°C)	T-RF
LA35F	*Brevibacterium*	ACCGGATACGACCATCTGC	166-184	57	147.3
LA35R	clone LA35	TCCCCAGTGTCAGTCACAGC	717-736	58	
SA19F	*Kineococcus*	TACGACTCACCTCGGCATC	163-181	56	158.9
SA19R	*spp.*	ACTCTAGTGTGCCCGTACCC	602-621	55	
SB37F	*Rhodoplanes*	AACGTGCCTTTTGGTTCG	143-160	56	142.9
SB37R	*spp.*	GCTCCTCAGTATCAAAGGCAG	616-626	55	
SA15F	*Pantoea*	CGATGTGGTTAATAACCGCAT	490-510	56	500.8
SA15R	*ananatis*	AAGCCTGCCAGTTTCAAATAC	668-688	55	

Table 3. Specificity of the poultry litter biomarker assay tested against fecal samples from within and outside the watershed.

Fecal sample (inside or outside watershed)	Number of samples tested (Number of samples containing potential biomarker)			
	Brevibacterium clone LA35	*Rhodoplanes* clone SB37	*Kineococcus* clone SA19	*Pantoea ananatis* clone SA15
Beef cattle (outside)	5 (0)	5 (2)	5 (1)	5 (0)
Beef cattle (inside)	5 (0)	5 (3)	5 (5)	5 (1)
Dairy cattle (outside)	2 (0)	2 (1)	2 (1)	2 (1)
Dairy cattle (inside)	1 (0)	1 (1)	1 (0)	1 (0)
Swine (outside)	1 (0)	1 (1)	1 (1)	1 (0)
Swine (inside)	1 (0)	1 (0)	1 (0)	1 (0)
Duck (outside)	2 (1)*	2 (2)	2 (2)	2 (2)
Duck (inside)	3 (0)	3 (1)	3 (1)	3 (2)
Goose (outside)	3 (1)*	3 (3)	3 (2)	3 (2)
Goose (inside)	2 (0)	2 (2)	2 (1)	2 (1)
Human sewage (outside)	2 (0)	2 (2)	2 (2)	2 (1)
Human sewage (inside)	4 (0)	4 (3)	4 (1)	4 (1)

* One duplicate amplified when analyzed with a nested PCR assay.

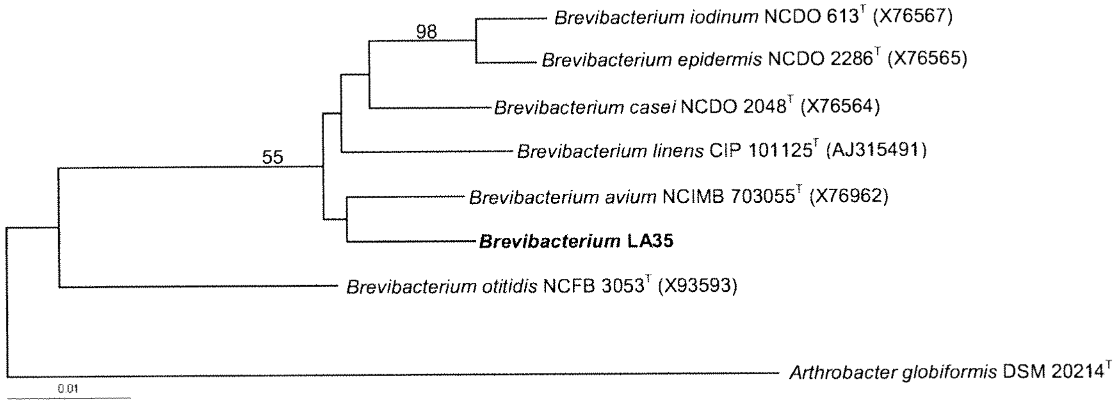

98
55
Brevibacterium iodinum NCDO 613^T (X76567)
Brevibacterium epidermis NCDO 2286^T (X76565)
Brevibacterium casei NCDO 2048^T (X76564)
Brevibacterium linens CIP 101125^T (AJ315491)
Brevibacterium avium NCIMB 703055^T (X76962)
Brevibacterium LA35
Brevibacterium otitidis NCFB 3053^T (X93593)
Arthrobacter globiformis DSM 20214^T
0.01

Figure 1. Reconstructed phylogentic tree of the *Brevibacterium* spp. based on 16S rRNA. Numbers at the nodes represent bootstrap values (i.e. the number of times this organism was found in this position relative to other organisms in 1000 resamplings of the data). Bootstraps less than 50% are not shown. The closest cultured organisms as reported in an NCBI BLAST search are reported. The distance bar represents a 1% estimated sequence divergence.

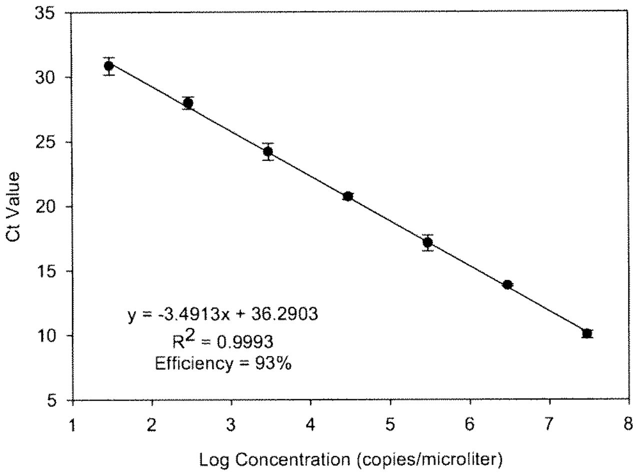


Figure 2. Standard curve of measured Ct values and standard deviations versus log plasmid biomarker concentration.

Table 4. Environmental samples tested for *Brevibacterium* clone LA35 poultry litter biomarker

Sample type	Number samples tested	% of samples containing biomarker [a]	% of samples quantifiable [b]	Range of biomarker present (16S rRNA copies/L water or g soil or g litter)
Litter	10	100	100	$2.2*10^{7} \pm 7.1*10^{6} - 2.5*10^{9} \pm 9.5*10^{7}$
Soil	10	100	50	$7.0*10^{3} \pm 4.4*10^{2} - 2.9*10^{5} \pm 2.0*10^{4}$
Edge of field runoff	10	100	100	$2.6*10^{3} \pm 1.2*10^{2} - 5.5*10^{7} \pm 5.3*10^{6}$
River	10	50	20	$2.9*10^{3} \pm 8.6*10^{2} - 3.2*10^{4} \pm 6.8*10^{3}$
Groundwater	6	0	0	Not applicable

[a] indicates the percent of samples in which the biomarker was identified by qPCR or nested qPCR methods

[b] indicates the percent of samples for which a quantifiable number of biomarker genes were measured by qPCR

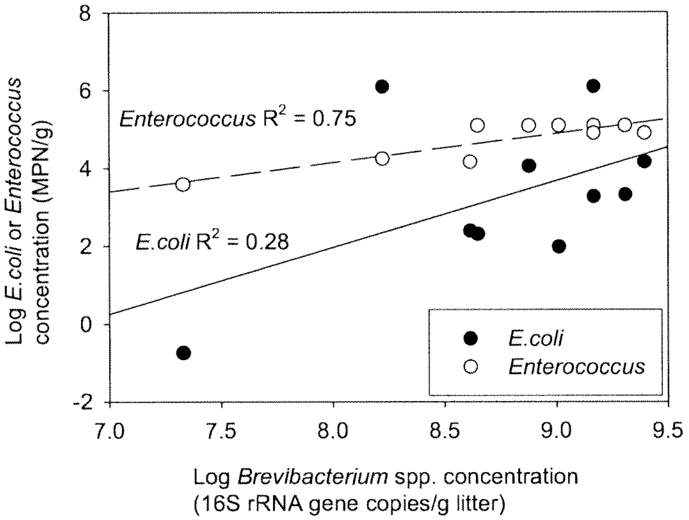


Figure 3. Correlation between the concentrations of poultry litter biomarker, *E. coli* and *Enterococcus spp.* in poulty litter samples.

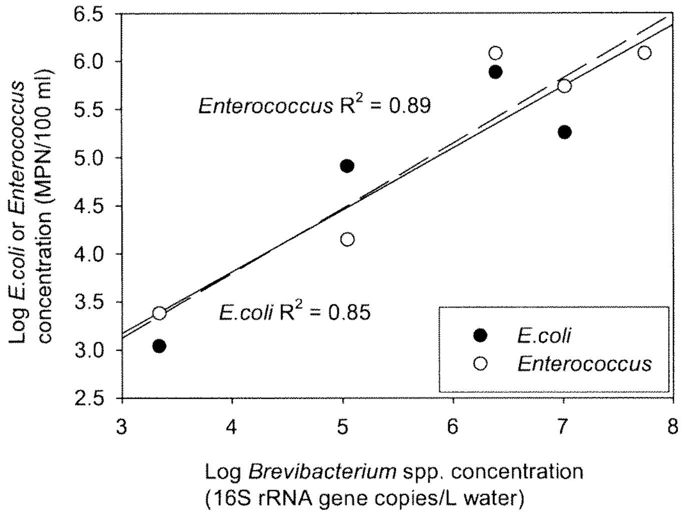


Figure 4. Correlation between the concentrations of poultry litter biomarker, *E. coli* and *Enterococcus* spp. in water samples.

Identification and Validation of a Poultry Litter-Specific Biomarker and Development of a 16S rRNA Based Quantitative PCR Assay

Jennifer L. Weidhaas[1], Tamzen W. Macbeth[1], Roger L. Olsen[2], Valerie J. Harwood[3, *]

1. North Wind, Inc. 1425 Higham Street, Idaho Falls, Idaho, 83402
2. CDM, 555 17th St., Suite 1100, Denver, CO 80202
3. * Department of Biology, University of South Florida, 4202 E. Fowler Ave., Tampa, Florida 33620, Phone: 813-974-1524, Fax: 813-974-3263 email vharwood@cas.usf.edu

Running title: Brevibacterium marker for fecal source tracking of poulty

ABSTRACT

A poultry litter-specific biomarker was developed for microbial source tracking (MST) in environmental waters. 16S rRNA sequences that were present in fecal-contaminated turkey and chicken litter were identified by terminal restriction fragment length polymorphism (T-RFLP). Cloning and sequencing of potential targets from pools of *E. coli, Bacteroides* or total bacterial DNA yielded four sequences that were ubiquitous in poultry litter and also contained unique sequences for development of target-specific PCR primers. Primer sensitivity and specificity were tested by nested PCR against ten composite poultry litter samples and fecal samples from beef and dairy cattle, swine, ducks, geese, and human sewage. The sequence with greatest sensitivity (100%) and specificity (93.5%) has 98% identity to *Brevibacterium avium*, and was detected in all litter samples. It was detected at low level in only one goose and one duck sample. A quantitative PCR assay was developed and tested on litter, soil and water samples. Litter concentrations were $2.2*10^7$ - $2.5*10^9$ gene copies/g. The biomarker was present in a majority of soil and water samples collected in and near areas where litter was spread, reaching concentrations of 2.9×10^5 gene copies·g^{-1} in soil samples and 5.5×10^7 gene copies·L^{-1} in runoff from the edges of fields. The biomarker will contribute to quantifying the impact of fecal contamination by land-applied poultry litter in this watershed. Furthermore, it has potential for determining fecal source allocations for total maximum daily load (TMDL) programs and ambient water quality assessment, and may be useful in other geographic regions.

INTRODUCTION

Excessive land application of poultry litter as a waste disposal mechanism has been linked to eutrophication of water bodies (28, 35, 39), the spread of pathogens (15, 19, 21), air and soil pollution with metals (11, 33) and groundwater contamination with nitrate (5). Despite these known effects, land application is still the typically practiced disposal method for poultry litter even though viable and economically favorable alternative disposal practices are available (7, 20).

Identification of the source of fecal pollution contaminating a watershed is of particular interest for protection of water resources and the safety of recreational waters. For example, TMDL assessments require identification of the source of contamination, which is also necessary for remediation of impaired waters(44). Current methods for detecting the presence of fecal pollution, which carries an increased risk of the presence of pathogenic microorganisms, involve the cultivation of fecal indicator organisms such as fecal coliforms in the family *Enterobacteriaceae* (Oklahoma Administrative Code, Title 785, Chapter 46). The U.S. EPA and many states recognize *Escherichia coli* and enterococci as indicators of freshwater recreational water quality (42).

Drawbacks to the use of indicator organisms which limit the ability of researchers to pinpoint sources of fecal contamination include the non-specificity of the fecal coliforms to one source (25, 43), variable survival rates of various indicator organisms (1) and the growth or extended persistence of these indicator organisms after release to the environment (12, 45). These drawbacks have lead to research into alternative methods for the assessment of human health risk

from microbial pathogens in recreational waters that do not include the culturing of fecal indicator organisms for identification and quantification of the source of fecal pollution (46).

A variety of microbial source tracking (MST) methods (for recent reviews see (17, 40, 47)) have been proposed as an alternative to cultivation of fecal coliforms. Some of these genotypic molecular based techniques have included library dependent methods (i.e., culture and isolate-based) such as ribotyping (10, 31) and repetitive element polymerase chain reaction (REP-PCR) (14). Library independent methods (i.e., detection of a genetic biomarker in extracted DNA) have also been developed using discovery techniques such as suspension arrays (8), subtractive hybridization (13, 26), and terminal restriction fragment length polymorphism (T-RFLP) (3), among others. Host marker specific targets have included *Enterococcus faecium* (37), *Bifidobacterium* and members of the *Bacteroidales* (3, 22, 38), among others. Relatively few microbial targets specific to poultry fecal material have been identified. To date *Enterococcus faecalis* (23), *E. coli* (10) and *Bacteriodes* (26) have been associated with poultry fecal material, but only the *Bacteroides* biomarker (26) was specifically associated with poultry and not other fecal sources The objective of this research was to identify a poultry litter-specific biomarker, validate its specificity against other sources of fecal material from within and outside the watershed and develop a 16S rRNA based real-time PCR assay for quantifying the biomarker in environmental samples. This work was carried out as part of ongoing litigation in which the plaintiff is the Oklahoma Attorney General.

METHODS

Sample collection. Litter samples were collected from ten separate facilities (poultry houses), nine chicken and one turkey facility. Litter samples were collected from 18 locations within each

poultry house through the entire depth of the litter. The subsamples (total volume of 4 to 5 gallons) from each house were composited, homogenized and split (riffle splitter) before placement into a sterile whirl pack (approximately 500 mL) and shipped on ice to the laboratory for analysis. Litter application areas in fields (soils) were sampled by collecting 20 subsamples on a predetermined grid pattern across a uniform subarea of one to ten acres in size. The zero to two inch sample from six inch soil cores were composited, disaggregated, sieved to 2 mm, ground, homogenized and split. Vegetation, feathers, and rocks were removed. The split soil samples (500 ml) were transported on ice to the laboratory. Nontarget fecal samples for specificity testing were collected as composites from groups of individuals (Table 3). Samples from beef cattle were collected from ten grazing fields, of which five were within the watershed and five were outside the watershed. Two independent duplicate samples were collected for each field, and each duplicate consisted of feces from ten scats. A total of 200 beef cattle scats were collected and composited into 20 samples. Duck and goose samples were collected in the same fashion, consisting of composites from ten individual scats, and independent duplicates were collected for each area. For ducks, three landing areas inside the watershed and two outside the watershed were sampled, while for geese, two landing areas inside and three landing areas outside the watershed were sampled. A total of 100 scats for duck and geese were collected and composited into 10 samples for duck and 10 samples for geese. Composite samples of fecal slurries were collected from swine facilities, one inside the watershed and one outside (2 duplicate samples) and dairy cattle facilities (one inside the watershed and two outside (2 duplicate samples each) human residential septic cleanout trucks (3 samples) and influent of three separate municipal wastewater treatment plants (3 samples). A total of 20 g of each fecal sample other than litter from each site was collected and was placed in a 20 ml, sterile,

polystyrene tube containing 10 ml of 20% glycerol and shipped on dry ice to the laboratory. All fecal samples were homogenized in the glycerol before DNA extraction. Discrete water samples from larger rivers and lakes were collected using a Van Dorn water sampler or with a churn splitter for discrete or composite samples. Samples from larger rivers were typically composites of 3 samples collected on a transect across the width of the river channel. Samples from smaller rivers were collected using automated samplers. Samples collected during high flow events were composited based on flow volume. Base flow samples were collected as grab samples. River samples were placed into sterile 1-L polystyrene bottles in duplicate and shipped on ice to the laboratory where they were filtered. Runoff samples from the litter application areas (e.g. edge of field runoff samples) were collected during or as soon as possible after rainfall events. Samples were collected either with a passive runoff collector for composite samples or with a dip sampler for discrete samples. Runoff samples were placed into sterile 1-L polystyrene bottles in duplicate and shipped on ice to the laboratory where they were filtered. Groundwater samples were collected directly from existing homeowner's wells or from hydraulically driven shallow probes. Spring samples were collected as grab samples or by using a peristaltic pump. All samples were placed into sterile 1-L polystyrene bottles and shipped onice to the laboratory where they were filtered.

Enumeration of Indicator Bacteria. Indicator bacteria (fecal coliforms, *E. coli* and enterococci) were enumerated according to standard methods using multiple tube fermentation (MTF) and calculation of the most probable number according to according to SM-9221F or SM-9230 (APHA, 2005). MTF tubes containing *E. coli* were identified using broth cultures supplemented with (MUG) (SM-9221F) (2).

Soil, Litter and Fecal Sample DNA Extraction. Genomic DNA was extracted from soil, liter and fecal samples with Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc.) following the manufacturer's instructions. Typically 0.25 g of soil or litter was used in each extraction. DNA was purified by size-exclusion chromatography. Sepharose CL-4B (Sigma-Aldrich) was resuspended in Tris-HCL and sterilized by autoclave at 121 ^{o}C for at least 20 minutes. Micro-bio spin columns (Bio-Rad Laboratories) were packed with 1 mL of Sepharose CL-4B through centrifugation. Sepharose columns were then washed twice with Tris-HCl buffer (pH 8) and 50 to 150 μl of sample was added. Purified DNA was concentrated with ethanol precipitation and re-eluted in 100 μL sterile water.

Water Sample DNA Extraction. Within 12 hours of receipt at the laboratory all water samples were filtered through a sterile Supor-200, 0.2 μM filter and frozen at -80°C. Filters were then shattered with sterile glass beads and vortexed vigorously for 15 minutes with sterile, DNase, and RNase free water to remove solids and cells from the filters. The cell suspension was removed from the centrifuge tubes by pipette and placed in a 2 mL bead beating tube from the Bio101 Fast®Spin® DNA extraction kits. The cells were centrifuged at 20,000 x g for 10 minutes, and the supernatant was decanted. Genomic DNA was then extracted using the Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc). The extracted DNA was quantified using a Nanodrop® UV-Vis Spectrophotometer.

T-RFLP Analysis. Extracted genomic DNA and/or cloned DNA was amplified with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM) labeled universal bacterial primers 8F-907R (16, 24), with *E.coli* genus specific primers (Tsen, et al. 1998), and *Bacteroidales* specific primers (Bernhard and Field, 2000). All PCR primers targeted the 16S rRNA gene. Triplicate PCR reactions were generated from each DNA extraction, combined and purified

using QIAquick PCR purification Kits (Qiagen). Approximately 200 ng each of PCR product was digested at 37°C for 6 hours with the *Msp*I restriction enzyme (20μ/μL) (New England BioLabs). Samples were denatured by heating to 95° C for 3 minutes followed by cooling to 4°C. The digested fragments were purified by ethanol precipitation.

Primer Design. Primers were designed using the ABI Primer Express v.2 program (Applied Biosystems, Foster City, CA) and were targeted to variable regions between the potential biomarker sequences and sequences of the top 20 closest related organisms in the GenBank database. The BLAST search (Basic Alignment Search Tool, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was used to check the specificity of each primer.

PCR Assay Conditions. PCR was used to amplify approximately 900 bp of the 16S rRNA genes from *Bacteria* for clone library construction. Each 25 μL PCR reaction included 0.4 mg mL $^{-1}$ molecular-grade bovine serum albumin (BSA) (Sigma Chemicals), 1X PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.5 μM of both the forward (8F) (16) and reverse (907R) (24) primer (Invitrogen), 1U Taq DNA polymerase (Promega), 0.2 mM dNTP (Invitrogen), 1 μL DNA template, and molecular-grade water (Promega). Amplification was performed on a PerkinElmer Model 9600 thermocycler using the following conditions: 94 °C for 5 minutes, 30 cycles of 94 °C (1 minute), 55 °C (45 seconds), and 72 °C (2 minute). A final extension at 72 °C for 7 minutes was performed and the PCR products were held at 4°C. Specificity of the PCR primers to the poultry litter biomarker was evaluated with nested PCR by first amplifying non-target fecal samples by universal bacterial primers 8F, 907R and then amplifying by the potential poultry litter biomarker PCR primers. The nested PCR master mix and thermocycler conditions were similar to the universal PCR with the following exceptions: 1) forward and reverse PCR

primers were specific to the potential poultry biomarker as shown in Table 2, 2) the annealing temperature was 60 °C. Amplification by nested PCR was evaluated by gel electrophoresis.

Clone Libraries. Clone libraries were constructed from the original genomic DNA extracted from the soil and litter samples and amplified with either universal bacterial primers 8F-907R (16, 24), targeting the 16S rRNA genes of *Bacteria* or the *E. coli* genus specific primers V1SF-V3AR (41). The TOPO ® Cloning Reaction methods from Invitrogen ™ were followed for clone library construction. Two clone libraries were constructed (targeting *Bacteria* and *E. coli*) from pooled DNA samples (i.e., 1 µl of genomic DNA extract from each sample was added to the PCR reaction for inclusion into the clones) based on the abundance of the various potential biomarkers as evidenced by the T-RFLP profiles.

qPCR Assay Conditions. Quantitative PCR (qPCR) was used to amplify 530 bp of the 16S rRNA gene from *Brevibacterium spp*. DNA samples were diluted to final concentrations of 3 ng/µL DNA. Each 25µL qPCR reaction included: 1X SYBR Green Master Mix (Roche), 0.5 µM of both the forward (LA35F) and reverse primer (LA35R) (Invitrogen), 5 % DMSO, 5 µL of diluted sample DNA, and molecular-grade water (Promega). Amplification was performed in triplicate on a Biorad Chromo4 thermocylcer using the following conditions: 50 °C for 2 minutes, 95 °C for 15 minutes, 45 cycles of 95 °C (30 seconds), 60 °C (30 seconds), and 72 °C (30 seconds) with a plate read. The 45 cycles was followed by a final extension at 50 °C for 5 minutes. Immediately following the final extension was a melting curve from 70 °C to 90 °C, by 0.1 degree increments, holding for 5 seconds with a plate read. DNA standards ranging from $6*10^{-15}$ to 10^{-21} ng/ul were prepared from serial dilutions of clone plasmid DNA containing the sequence of interest and used to develop the standard curve and method detection limit. Gene copy numbers were calculated from concentrations of positive control standards assuming 9.124

* 10^{14} bp/ul of DNA and one gene copy per genome. Detection limits for the qPCR assay were approximately 2000 plasmid copies in *E. coli*/L water and 7.3 *10^4 plasmid copies in *E. coli*/gram of soil. Nested qPCR was performed by first amplifying DNA with the universal bacterial 16S rRNA 8F (16) and 907R (24) primers. The production of PCR products was confirmed on a 1.5% agarose gel. The 16S rRNA PCR products were purified with the QIAquick PCR purification kit (QIAGEN) were subjected to qPCR as previously described using the LA35F and LA35R primers for the poultry litter biomarker.

Phylogeny. The phylogeny of the LA35 clone was investigated using the following methods. The clone sequences were assembled and aligned with BioEdit v. 7.0.5.3 and sequences were checked for chimeras with the Ribosomal Database Project II Chimera Check program and Bellerophon. The 16S rRNA sequences of the closest neighbors to the clone sequences were downloaded for inclusion in the phylogenic analysis. Multiple sequence alignments were constructed with Clustal W alignment tool and manually aligned in BioEdit. The bootstraps (1000 resamplings), maximum likelihood and distance matrix analysis (Kimura), and the reconstruction of the phylogenetic trees (FITCH) were performed with the Phylip 3.65 package and in particular the programs SEQBOOT, DNAML, DNADIST, FITCH, CONSENSE, and RETREE. The reconstructed phylogenetic tree was visualized with PhyloDraw V. 0.8 (Graphics Application Lab, Pusan National University).

RESULTS

Identification of potential biomarkers by T-RFLP. A total of 20 T-RFLP profiles were generated from the 5 subsamples of each of the two litter and two soil samples. The T-RFs common among the subsamples and representing more than 1% of the community were selected

for cloning and sequencing (Table 1). A total of 3 *E. coli* T-RFs (i.e., T-RF 496.0, 498.9 and 500.8) and 3 *Bacteria* T-RFs (i.e., T-RF142.9, 147.3 and 158.9) were selected for cloning and sequencing. Clone libraries were constructed from PCR products amplified with *E. coli* specific primers (V1SF-V3AR) (41) or universal bacterial primers (8F-907R) (16, 24). A total of 300 plasmids from the clone libraries were randomly picked. T-RFLP analysis was carried out on each plasmid insert to identify which plasmids contained the T-RFs of potential biomarkers. Inserts containing the T-RFs of interest were sequenced and PCR primers were developed for those sequences containing mismatches as compared to BLAST database results of the top 20 closely related organisms. In all 4 PCR primers for members of 4 genera were developed; a *Brevibacterium spp.*, a *Rhodoplanes spp.*, a *Kineococcus spp.* and a *Pantoea ananatis* strain (Table 2). Two *E. coli* T-RFs were from plasmids that did not contain mismatches between the sequence of interest and the sequences of closely related organisms identified in a BLAST search and therefore were not appropriate biomarkers.

Evaluation of biomarkers against fecal samples. The PCR assays developed for the 4 potential biomarkers of poultry litter were tested for amplification against a variety of nontarget fecal samples from within and outside the watershed (Table 3). Only the *Brevibacterium* clone LA35 appeared to be a potential candidate biomarker for poultry litter in that did not amplify in any fecal samples with the exception of weak amplification in one duck and one goose sample from outside the watershed when analyzed with a nested PCR approach (i.e. PCR with universal bacterial primers and then with the *Brevibacterium* clone LA35 primers). The reconstructed phylogenetic tree of the *Brevibacterium* clone LA35 in relationship to other *Brevibacterium* spp. is presented in Figure 1.

Quantification of the poultry litter biomarker in environmental samples. A SYBR green qPCR protocol was developed and optimized using the LA35F and LA35R primers (Table 2) specific to the *Brevibacterium* clone LA35 poultry litter biomarker. The standard curve of the qPCR assay for the biomarker is presented in Figure 2. The detection limit of the qPCR assay was 6 gene copies/ul of extracted DNA.

Environmental samples from the potential poultry litter impacted watershed were tested for the presence of the biomarker with the qPCR assay (Table 4). A variety of samples from within the watershed were tested, some of which were expected to contain the biomarker (e.g., litter, contaminated soil, runoff samples), some of which had variable potential for higher biomarker levels (e.g., surface water), and some of which had lower potential for biomarker presence (i.e., groundwater samples).

The correlation between the poultry litter biomarker concentration (i.e., as quantified by qPCR) in water and litter samples and *E. coli* and *Enterococcus* as measured by most probable number is presented in Figures 3 and 4. In general the *Enterococcus* MPN counts were well correlated with the concentration of the biomarker in litter ($R^2 = 0.75$) and with the biomarker concentration in water samples ($R^2 = 0.89$). The correlation between *E. coli* concentrations and the biomarker in water samples was also strong ($R^2 = 0.85$) while *E. coli* was less tightly (but significantly) correlated with the biomarker in litter samples ($R^2 = 0.28$). Correlation of the biomarker with *E. coli* and *Enterococcous* spp. provides a line of evidence of the human health risk associated with the runoff from poultry litter application to fields although there is evidence that regrowth of these organisms is possible once they are introduced into the environment (36).

DISCUSSION

The *Brevibacterium* sp. poultry litter biomarker developed in this study was validated in terms of sensitivity (100%) against numerous positive (poultry litter) samples from different locations with the watershed and for specificity (93.5%) against composite non-target fecal samples. These practices are in accordance with recent critical reviews (34, 40) that strongly recommend MST method validation. Future efforts will attempt to extend the method validation outside the watershed and possible outside the region as this biomarker could be useful for identifying fecal pollution sources in other river systems and coastal waters.

The *Brevibacterium* clone LA35 poultry litter biomarker was most closely related to *Brevibacterium avium*, which is associated with bumble-foot lesions in poultry (32). *Brevibacterium* spp. were recently identified in spent mushroom compost that was originally derived from chicken litter and cereal straw (29). Additionally *Brevibacterium avium, Brevibacterium iodinum,* and *Brevibacterium epidermidis* were found to represent more than 7% of a 16S rRNA clone library originating from broiler chicken litter (27). Certain *Brevibacterium* spp. are associated with milk and cheese curds(6), human skin(9), and soils (30). *Brevibacterium* spp. have been associated with disease in humans although to date these opportunistic pathogens have only been isolated from immunocompromised patients (4, 9, 18).

As poultry litter is land-applied as a disposal practice (19, 33, 35), it was important to identify a marker that could survive the process of deposition on bedding and spreading on fields. Therefore, the T-RFLP screening process included both litter and contaminated soil samples. This strategy allowed for the rapid elimination of numerous targets that could be abundant in the poultry fecal material, but not as abundant in the litter and not present in the environment after

litter application. This strategy for marker identification is in contrast with the work by Lu and colleagues (2007) where a genome fragment enrichment method was used to identify microbial sequences specific to chicken feces. Based on the PCR assays developed from clone libraries of the genome fragments, 6 to 40% of the chicken fecal samples collected from a wide geographic region contained DNA that could be amplified by the various assays (26). In comparison the LA35 biomarker was found in all the poultry litter samples tested, although it should be noted that all of the samples were collected in the Oklahoma/Arkansas region.

The examination of environmental samples from within the poultry litter impacted watershed suggest a correlation between the application of poultry litter to a field and concentration of the biomarker in the receiving waters, as evidenced by the generally decreasing trend in biomarker concentration with decreasing concentration of fecal indicator organisms. These results indicate that the watershed is in fact being impacted by the application of poultry litter to fields within the watershed. However, the magnitude of the impact as measured by the distribution of the biomarker within the watershed cannot be quantified with the limited number of environmental samples processed to date. Future work will include the testing of environmental samples from within the watershed by the qPCR assay to evaluate the distribution of the poultry litter-specific biomarker as compared to indicator bacteria, antibiotics and heavy metals. Additionally, testing of the poultry litter-specific biomarker against more fecal samples from other watersheds and additional avian fecal material will be conducted as the LA35 poultry litter biomarker was found in low abundance (i.e., a nested PCR approach was required for detection) in two non-target composite avian fecal samples (i.e., a duck and a goose sample) from outside the watershed.

Conclusions

In summary a novel biomarker of poultry litter was identified and a 16S rRNA based real-time PCR assay was developed for this biomarker. The specificity of the assay (93.5%) was tested against 31 separate non-target fecal samples and sensitivity was tested against10 target litter samples (100%). The field applicability of the assay was evaluated by testing for the biomarker in environmental samples expected to have variable concentrations of the biomarker, which we hypothesized would be correlated with the concentration of fecal indicator bacteria. A generally positive correlation was found between biomarker concentration and fecal indicator bacteria concentration which was particularly strong for enterococci. The research presented herein is the first identification of a *Brevibacterium* spp. for microbial source tracking studies and is among the first quantifiable method for tracking of poultry fecal sources in environmental waters.

ACKNOWLEDGMENTS

This research was conducted in connection with work performed as retained experts in a pending legal case brought by the State of Oklahoma against several poultry integrators. Drs. Harwood & Olsen have been retained to serve as expert witnesses by the State of Oklahoma and have provided testimony regarding this research.

The authors are grateful for the assistance provided by Kyle Collins, William Blackmore, James Jackson, Erin O'Leary Jeapson and Michelle Andrews. Additionally the authors acknowledge the Molecular Research Core Facility at Idaho State University for graciously allowing us the use of their laboratory space and equipment.

References

1. **Anderson, K., J. Whitlock, and V. Harwood.** 2005. Persistence and Differential Survival of Fecal Indicator Bacteria in Subtropical Waters and Sediments. Applied and Environmental Microbiology **71:**3041-3048.
2. **APHA.** 2005. Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association, Inc., Washington, D.C.
3. **Bernhard, A., and K. Field.** 2000. Identification of Nonpoint Sources of Fecal Pollution in Coastal Waters by Using Host-Specific 16S Ribosomal DNA Genetic Markers from Fecal Anaerobes. Applied and Environmental Microbiology **66:**1587-1594.
4. **Beukinga, I., H. Rodriguez-Villalobos, A. Deplano, F. Jacobs, and M. Struelens.** 2005. Management of long-term catheter-related *Brevibacterium* bacteraemia. Clinical Microbiology and Infection **10:**465-467.
5. **Bitzer, C., and J. Sims.** 1988. Estimating the availability of nitrogen in poultry manure through laboratory and field studies. Journal of Environmental Quality **17:**47-54.
6. **Brennan, N., A. Ward, T. Beresford, P. Fox, M. Goodfellow, and T. Cogan.** 2002. Biodiversity of the bacterial flora on the surface of a smear cheese. Applied and Environmental Microbiology **68:**820-830.
7. **Bujozcek, G., J. Oleszkiewicz, R. Sparling, and S. Cenkowski.** 2000. High Solid Anaerobic Digestion of Chicken Manure. Journal of Agricultural Engineering Research **76:**51-60.
8. **Call, D., D. Satterwhite, and M. Soule.** 2007. Using DNA suspension arrays to identify library-independent markers for bacterial source tracking. Applied and Environmental Microbiology **41:**3740-3746.

9. **Cannon, J., F. Spadoni, S. Pesh-Iman, and S. Johnson.** 2005. Pericardial infection caused by *Brevibacterium casei*. Clinical Microbiology and Infection **11:**164-165.

10. **Carson, C., B. Shear, M. Ellersieck, and A. Asfaw.** 2001. Identification of Fecal *Escherichia coli* from Humans and Animals by Ribotyping. Applied and Environmental Microbiology **67:**1503-1507.

11. **Connor, R., M. Connor, K. Irgolic, J. Sabrsula, H. Gurleyuk, R. Brunette, C. Howard, J. Garcia, J. Brien, J. Brien, and J. Brien.** 2005. Transformations, Air Transport and Human Impact of Arsenic from Poultry Litter. Environmental Forensics **6:**83-89.

12. **Desmarais, T., H. Solo-Gabriele, and C. Palmer.** 2002. Influence of Soil on Fecal Indicator Organisms in a Tidally Influenced Subtropical Environment. Applied and Environmental Microbiology **68:**1165-1172.

13. **Dick, L. K., A. E. Bernhard, T. J. Brodeur, J. W. Santo Domingo, J. M. Simpson, S. P. Walters, and K. G. Field.** 2005. Host distributions of uncultivated fecal *Bacteroidales* bacteria reveal genetic markers for fecal source identification. Applied and Environmental Microbiology **71:**3184-3191.

14. **Dombek, P., L. Johnson, S. Zimmerley, and M. Sadowsky.** 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. Applied and Environmental Microbiology **66:**2572-2577.

15. **Edwards, D., and T. Daniel.** 1994. A comparison of runoff quality effects of organic and inorganic fertilizers applied to fescue grass plots. Journal of the American Water Resources Association **30:**35-41.

16. **Edwards, U., T. Rogall, H. Blocker, M. Emde, and E. Bottger.** 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Research **17:**7843-7853.

17. **Field, K., and M. Samadpour.** 2007. Fecal source tracking, the indicator paradigm, and managing water quality. Water Research **41:**3517-3538.

18. **Janda, W., P. Tipirneni, and R. Novak.** 2003. *Brevibacterium casei* Bacteremia and Line Sepsis in a Patient with AIDS. Journal of Infection **46:**61-64.

19. **Jenkins, M., D. Endale, H. Schomber, and R. Sharpe.** 2006. Fecal bacteria and sex hormones in soil and runoff from cropped watersheds amended with poultry litter. Science of the Total Environment **358:**164-177.

20. **Kelleher, B., J. Leahy, A. Henihan, T. O'Dwyer, D. Sutton, and M. Leahy.** 2002. Advances in poultry litter disposal technology – a review. Bioresource Technology **83:**27-36.

21. **Kelley, T., O. Pancorbo, W. Mercka, S. Thompson, M. Cabrera, and H. Barnhart.** 1994. Fate of Selected Bacterial Pathogens and Indicators in Fractionated Poultry Litter During Storage. Journal of Applied Poultry Research **3:**279-288.

22. **Kildare, B. J., C. M. Leutenegger, B. S. McSwain, D. G. Bambic, V. B. Rajal, and S. Wuertz.** 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. Water Research **41:**3701-3715.

23. **Kuntz, R., P. Hartel, J. Rodgers, and W. Segars.** 2004. Presence of *Enterococcus faecalis* in broiler litter and wild bird feces for bacterial source tracking. Water Research **38:**3551-3557.

24. **Lane, D.** 1991. 16S/23S rRNA sequencing. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid sequencing techniques in bacterial systematics. John Wiley and Sons, New York, N.Y.

25. **Leclerc, H., D. Mossel, S. Edberg, and C. Struijk.** 2001. Advances in the Bacteriology of the Coliform Group: Their Suitability as Markers of Microbial Water Safety. Annual Reviews in Microbiology **55:**201-234.

26. **Lu, J., J. Domingo, and O. Shanks.** 2007. Identification of a chicken-specific fecal microbial sequences using a metagenomic approach. Water Research **41:**3561-3574.

27. **Lu, J., S. Sanchez, C. Hofacre, J. Maurer, B. Harmon, and M. Lee.** 2003. Evaluation of Broiler Litter with Reference to the Microbial Composition as Assessed by Using 16S rRNA and Functional Gene Markers. Applied and Environmental Microbiology **69:**901-908.

28. **Mozaffari, M., and J. Sims.** 1994. Phosphorus availability and sorption in an Atlantic coastal plain watershed dominated by animal-based agriculture. Soil Science **157:**97-107.

29. **Ntougias, S., G. Zervakis, N. Kavroulakis, C. Ehaliotis, and K. Papadopoulou.** 2004. Bacterial Diversity in Spent Mushroom Compost Assessed by Amplified rDNA Restriction Analysis and Sequencing of Cultivated Isolates. Systematic and Applied Microbiology **27:**746-754.

30. **Onraedt, A., W. Soetaert, and E. Vandamme.** 2005. Industrial importance of the genus *Brevibacterium*. Biotechnology Letters **27:**527-533.

31. **Parveen, S., R. Murphree, L. Edmiston, C. Kaspar, and M. Tamplin.** 1999. Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human

and nonhuman sources of fecal pollution. Applied and Environmental Microbiology **65:**3142-3147.

32. **Pascual, C., and M. Collins.** 1999. *Brevibacterium avium* sp. nov., isolated from poultry. International Journal of Systematic Bacteriology **49:**1527-1530.

33. **Pirani, A., K. Brye, T. Daniel, B. Haggard, E. Gbur, and J. Mattice.** 2006. Soluble Metal Leaching from a Poultry Litter–Amended Udult under Pasture Vegetation. Vadose Zone Journal **5:**1017-1034.

34. **Santo Domingo, J., D. Bambic, T. Edge, and S. Wuertz.** 2007. Quo vadis source tracking? Towards a strategic framework for environmental monitoring of fecal pollution. Water Research **41:**3539-3552.

35. **Schroeder, P., D. Radcliffer, and M. Cabrera.** 2004. Rainfall Timing and Poultry Litter Application Rate Effects on Phosphorus Loss in Surface Runoff. Journal of Environmental Quality **33:**2201-2209.

36. **Scott, T., J. Rose, T. Jenkins, S. Farrah, and J. Lukasik.** 2002. Microbial Source Tracking: Current Methodology and Future Directions. Applied and Environmental Microbiology **68:**5796-5803.

37. **Scott, T. M., T. M. Jenkins, J. Lukasik, and J. B. Rose.** 2005. Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. Environmental Science and Technology **39:**283-287.

38. **Seurinck, S., T. Defoirdt, W. Verstraete, and S. D. Siciliano.** 2005. Detection and quantification of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. Environmental Microbiology **7:**249-259.

39. **Sharpley, A., T. Daniel, J. Sims, and D. Pote.** 1996. Determining environmentally sound soil phosphorus levels. Journal of Soil and Water Conservation **51:**160-168.

40. **Stoeckel, D., and V. Harwood.** 2007. Performance, design and analysis in microbial source tracking studies. Applied and Environmental Microbiology **73:**2405-2415.

41. **Tsen, H., C. Lin, and W. Chi.** 1998. Development and use of 16S rRNA gene targeted PCR primers for the identification of *Escherichia coli* cells in water. Journal of Applied Microbiology **85:**554-560.

42. **USEPA.** 2000. Improved enumeration methods for the recreational water quality indicators: enterococci and *Escherichia coli.* EPA-821/R-771 97/004. U.S. Environmental Protection Agency.

43. **USEPA.** 2005. Microbial source tracking guide document, EPA/600/R-05/064. U.S. Environmental Protection Agency.

44. **USEPA.** 2001. Protocol for developing pathogen TMDLs. EPA 841-R-00-002. U.S. Environmental Protection Agency.

45. **Van Donsel, D., E. Geldreich, and N. Clarke.** 1967. Seasonal Variations in Survival of Indicator Bacteria in Soil and their Contribution to Storm-water Pollution. Applied Microbiology **15:**1362-1370.

46. **Wade, T., R. Calderon, E. Sams, M. Beach, K. Brenner, A. Williams, and A. Dufour.** 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspectives **114:**24-28.

47. **Wuertz, S., and J. Field.** 2007. Emerging microbial and chemical source tracking techniques to identify origins of fecal contamination in waterways. Water Research **41:**3515-3516.

Table 1. Common T-RFs among replicates from two fecal-contaminated poulty litter samples and two soils to which the litter had been applied.

	Number of subsamples tested (number containing T-RF of interest)			
T-RF	Litter A	Litter B	Soil A	Soil B
E.coli PCR products, digested with *MspI*				
496.0	4 (4)	5 (4)	5 (3)	5 (5)
498.9	4 (4)	5 (5)	5 (4)	5 (5)
500.8	4 (4)	5 (5)	5 (5)	5 (5)
Universal bacteria PCR products, digested with *MspI*				
80.1	4 (4)	5 (5)	5 (0)	3 (3)
130.9	4 (3)	5 (5)	5 (1)	3 (0)
142.9	4 (4)	5 (4)	5 (2)	3 (2)
147.3	4 (4)	5 (5)	5 (5)	3 (2)
158.9	4 (3)	5 (5)	5 (4)	3 (2)
165.0	4 (3)	5 (5)	5 (4)	3 (2)

*Underlined T-RFs correlate to those organisms for which PCR primers were developed

Table 2. Nucleotide sequences and targets of primers used in this study.

Primer	Target	Sequence (5'-3')	Position	Tm (°C)	T-RF
LA35F	*Brevibacterium*	ACCGGATACGACCATCTGC	166-184	57	147.3
LA35R	clone LA35	TCCCCAGTGTCAGTCACAGC	717-736	58	
SA19F	*Kineococcus*	TACGACTCACCTCGGCATC	163-181	56	158.9
SA19R	*spp.*	ACTCTAGTGTGCCCGTACCC	602-621	55	
SB37F	*Rhodoplanes*	AACGTGCCTTTTGGTTCG	143-160	56	142.9
SB37R	*spp.*	GCTCCTCAGTATCAAAGGCAG	616-626	55	
SA15F	*Pantoea*	CGATGTGGTTAATAACCGCAT	490-510	56	500.8
SA15R	*ananatis*	AAGCCTGCCAGTTTCAAATAC	668-688	55	

Table 3. Specificity of the poultry litter biomarker assay tested against fecal samples from within and outside the watershed.

Fecal sample (inside or outside watershed)	Number of samples tested (Number of samples containing potential biomarker)			
	Brevibacterium clone LA35	*Rhodoplanes* clone SB37	*Kineococcus* clone SA19	*Pantoea ananatis* clone SA15
Beef cattle (outside)	5 (0)	5 (2)	5 (1)	5 (0)
Beef cattle (inside)	5 (0)	5 (3)	5 (5)	5 (1)
Dairy cattle (outside)	2 (0)	2 (1)	2 (1)	2 (1)
Dairy cattle (inside)	1 (0)	1 (1)	1 (0)	1 (0)
Swine (outside)	1 (0)	1 (1)	1 (1)	1 (0)
Swine (inside)	1 (0)	1 (0)	1 (0)	1 (0)
Duck (outside)	2 (1)*	2 (2)	2 (2)	2 (2)
Duck (inside)	3 (0)	3 (1)	3 (1)	3 (2)
Goose (outside)	3 (1)*	3 (3)	3 (2)	3 (2)
Goose (inside)	2 (0)	2 (2)	2 (1)	2 (1)
Human sewage (outside)	2 (0)	2 (2)	2 (2)	2 (1)
Human sewage (inside)	4 (0)	4 (3)	4 (1)	4 (1)

* One duplicate amplified when analyzed with a nested PCR assay.

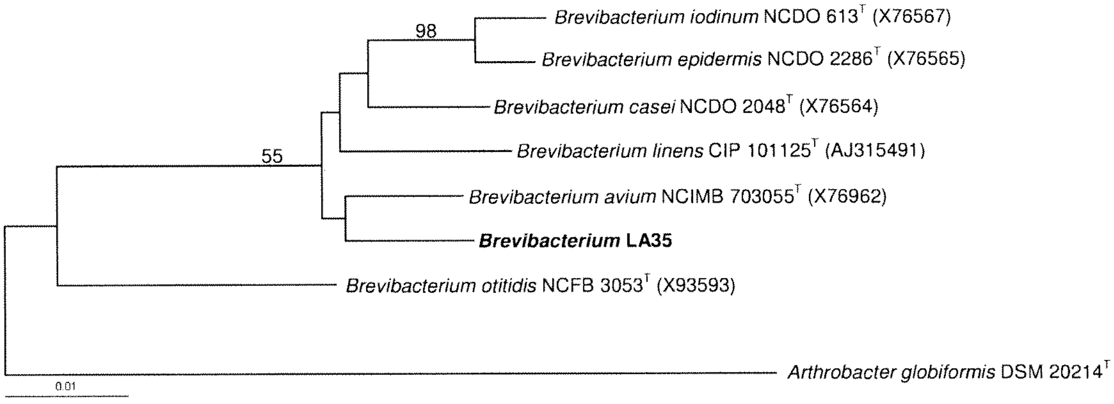

98
Brevibacterium iodinum NCDO 613^T (X76567)
Brevibacterium epidermis NCDO 2286^T (X76565)
Brevibacterium casei NCDO 2048^T (X76564)
55
Brevibacterium linens CIP 101125^T (AJ315491)
Brevibacterium avium NCIMB 703055^T (X76962)
***Brevibacterium* LA35**
Brevibacterium otitidis NCFB 3053^T (X93593)
Arthrobacter globiformis DSM 20214^T
0.01

Figure 1. Reconstructed phylogentic tree of the *Brevibacterium* spp. based on 16S rRNA. Numbers at the nodes represent bootstrap values (i.e. the number of times this organism was found in this position relative to other organisms in 1000 resamplings of the data). Bootstraps less than 50% are not shown. The closest cultured organisms as reported in an NCBI BLAST search are reported. The distance bar represents a 1% estimated sequence divergence.

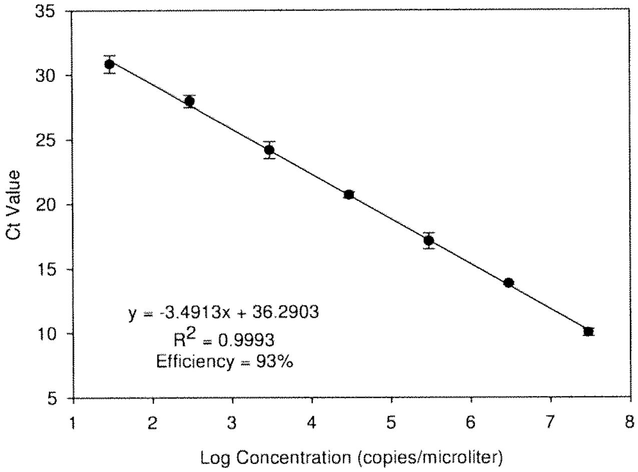


Figure 2. Standard curve of measured Ct values and standard deviations versus log plasmid biomarker concentration.

Table 4. Environmental samples tested for *Brevibacterium* clone LA35 poultry litter biomarker

Sample type	Number samples tested	% of samples containing biomarker [a]	% of samples quantifiable [b]	Range of biomarker present (16S rRNA copies/L water or g soil or g litter)
Litter	10	100	100	$2.2*10^{7} \pm 7.1*10^{6} - 2.5*10^{9} \pm 9.5*10^{7}$
Soil	10	100	50	$7.0*10^{3} \pm 4.4*10^{2} - 2.9*10^{5} \pm 2.0*10^{4}$
Edge of field runoff	10	100	100	$2.6*10^{3} \pm 1.2*10^{2} - 5.5*10^{7} \pm 5.3*10^{6}$
River	10	50	20	$2.9*10^{3} \pm 8.6*10^{2} - 3.2*10^{4} \pm 6.8*10^{3}$
Groundwater	6	0	0	Not applicable

[a] indicates the percent of samples in which the biomarker was identified by qPCR or nested qPCR methods

[b] indicates the percent of samples for which a quantifiable number of biomarker genes were measured by qPCR

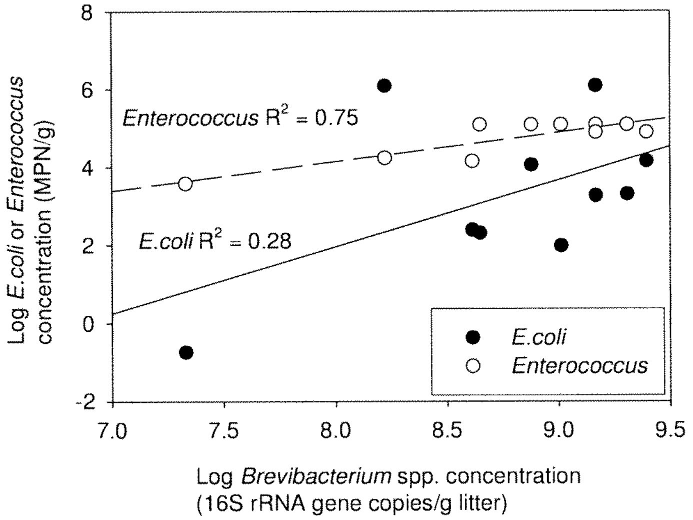


Figure 3. Correlation between the concentrations of poultry litter biomarker, *E. coli* and *Enterococcus spp.* in poulty litter samples.

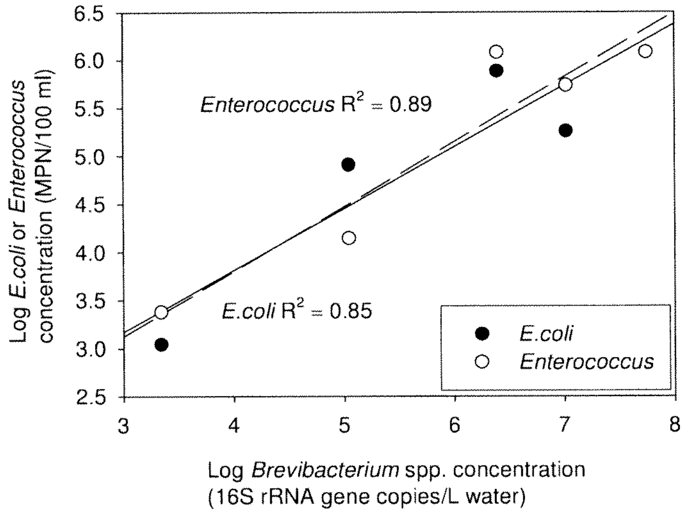


Figure 4. Correlation between the concentrations of poultry litter biomarker, *E. coli* and *Enterococcus* spp. in water samples.

North Wind

CDM

Identification and Validation of a Novel Poultry Litter Biomarker for Tracking Fecal Pollution

USF UNIVERSITY OF SOUTH FLORIDA

T.W. Macbeth[1], J.L. Weidhaas[1], R.L. Olsen[2], K.S. Sorenson[2], and V.J. Harwood[3]
1. North Wind, Inc. (Idaho Falls, ID) 2. CDM (Denver, CO) 3. Department of Biology, University of South Florida

Introduction

Identifying sources of fecal pollution contaminating watershed is of particular interest for protecting water resources and the safety of recreational waters. Current methods for detecting the presence of fecal pollution, involve the cultivation of fecal indicator organisms, such as fecal coliforms in the family *Enterobacteriaceae*. Drawbacks to the use of indicator organisms include the non-specificity of the fecal coliforms to one source (7, 9), variable survival rates of indicator organisms (1) and the growth or extended persistence of these indicator organisms after release to the environment (4, 10). These drawbacks have lead to research into non-culture based alternative methods, such as molecular methods, for the assessment of human health risk from fecal pollution in recreational waters. This research focused on the identification and quantification of fecal indicator organisms or biomarkers, in order to evaluate impacts of fecal pollution (11).

The objective of this research was to identify a poultry litter-specific biomarker, validate its specificity against other sources of fecal material from within and outside a watershed impacted by fecal pollution and develop a 16S rRNA-based real-time PCR assay for quantifying the biomarker in environmental samples.

Terminal restriction fragment length polymorphism (T-RFLP) was used to screen for 16S rRNA sequences that were present in fecal-contaminated poultry litter and soils to which litter was applied. Cloning and sequencing of potential targets from pools of *E. coli*, *Bacteroides* or total bacterial DNA yielded four sequences that were ubiquitous in poultry litter and also contained unique sequences for development of target-specific PCR primers. PCR primers for these potential poultry litter biomarkers were tested against nontarget fecal samples. A *Brevibacterium* sp. was found to be unique to poultry litter, and a real-time quantitative PCR assay was developed for this biomarker.

Sample Collection- Samples were aseptically collected from litter (10 poultry houses), soils to which litter was applied (129 samples), nontarget fecal samples (36 samples), discrete water sample from rivers and lakes (39 samples), groundwater (39 samples), runoff from litter application areas (47 samples), and clean poultry bedding (3 samples).

DNA Extraction- Genomic DNA was extracted using the Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc.). A follow on clean up step with Sepharose CL-4B (Sigma-Aldrich) was conducted if PCR inhibition was encountered.

Enumeration of Indicator Bacteria- Indicator bacteria (fecal coliforms, *E. coli* and enterococci) were enumerated according to standard methods using multiple tube fermentation (MTF) and calculation of the most probable number according to SM-9221F or SM-9230 (1).

T-RFLP Analysis- Extracted genomic DNA and/or clone DNA was amplified with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM) labeled universal bacterial primers 8F-907R(5, 6), with *E.coli* genus specific primers VISF-V3AR(8), and *Bacteroides* genus specific primers (2). All PCR primers targeted the 16S rRNA gene.

Phylogeny- The phylogeny of the *Brevibacterium* sp. was investigated using the following programs: BioEdit V. 7.0.5.3, RDP II, Bellerophon, Clustal W, Philip 3.65 and Phylodraw V.0.8.

Clone Libraries- Clone libraries were constructed from the original genomic DNA extracted from the soil and litter samples and amplified with either universal bacterial primers 8F-907R (5, 6) targeting the 16S rRNA genes of *Bacteria* or the *E. coli* genus specific primers VISF-V3AR (8). Two clone libraries were constructed (targeting *Bacteria* and *E. coli*) from pooled DNA samples based on the abundance of the various potential biomarkers as evidenced by the T-RFLP profiles.

qPCR Assay- qPCR, using SYBR Green Master Mix (Roche) and the LA35 primer, was used to amplify 530 bp of the 16S rRNA gene from *Brevibacterium* sp. Minimum detection limit for the qPCR assay was 6-16S rRNA gene copies/µL DNA extraction.

Results and Discussion

IDENTIFYING POTENTIAL BIOMARKER BY T-RFLP- Clone libraries were constructed from PCR products amplified with *E. coli* specific primers or universal bacterial primers. T-RFLP analysis was performed on each plasmid insert to identify which plasmids contained the T-RFs of potential biomarkers (Table 1). Four PCR primers for members of 4 genera were developed; a *Brevibacterium* sp., a *Rhodoplanes* sp., a *Kineococcus* sp. and a *Pantoea ananatis* strain.

An NCBI database search of organisms with >97 % similarity to the 4 clone sequences was conducted to determine if any sequences were associated with poultry litter or animal or human feces. The NCBI search revealed that only 2 of 19 organisms in the database that were closely related to the *Brevibacterium* sp. clone were associated with poultry litter, 2 of 50 *Pantoea* sp. clones were related to human feces and none of the *Kineococcus* sp. or *Rhodoplanes* sp. clones were associated with poultry, animal or human feces.

Table 1. Common T-RFs among replicates

T-RF	Number of subsamples digested (number containing T-RF of interest)			
	Litter A	Litter B	Soil A	Soil B
E. coli PCR products, digested with *Msp*I				
496.0	4 (4)	5 (4)	5 (3)	5 (5)
496.9	4 (4)	5 (5)	5 (4)	5 (5)
520.8	4 (4)	5 (5)	5 (5)	5 (5)
Universal bacteria PCR products, digested with *Msp*I				
80.1	4 (4)	5 (5)	5 (0)	3 (3)
130.9	4 (3)	5 (5)	5 (1)	3 (0)
142.9	4 (4)	5 (4)	5 (2)	3 (2)
147.3	4 (4)	5 (5)	5 (5)	3 (2)
158.9	4 (3)	5 (5)	5 (4)	3 (2)
165.0	4 (3)	5 (3)	5 (4)	3 (2)

*Underlined T-RFs correlate to those organisms for which PCR primers were developed.

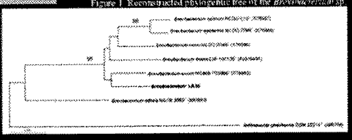
Fecal sample (inside or outside watershed)	Number of samples tested (Number of samples containing potential biomarker)			
	Brevibacteria clone LA35	*Rhodoplanes* clone SB37	*Kineococcus* clone SA15	*Pantoea ananatis* clone SA13
Beef cattle (outside)	5 (0)	5 (2)	5 (1)	5 (0)
Beef cattle (inside)	5 (0)	5 (3)	5 (0)	5 (1)
Dairy cattle (outside)	2 (0)	2 (1)	2 (1)	2 (1)
Dairy cattle (inside)	1 (0)	1 (1)	1 (0)	1 (0)
Swine (outside)	1 (0)	1 (1)	1 (1)	1 (0)
Swine (inside)	1 (0)	1 (0)	1 (0)	1 (0)
Duck (outside)	2 (1)*	2 (2)	2 (2)	2 (2)
Duck (inside)	3 (0)	3 (1)	3 (1)	3 (2)
Goose (outside)	3 (1)*	3 (3)	3 (2)	3 (2)
Goose (inside)	2 (0)	2 (2)	2 (1)	2 (1)
Human (outside)	2 (0)	2 (2)	2 (2)	2 (1)
Human (inside)	4 (0)	4 (3)	4 (1)	4 (1)

* One duplicate amplified when analyzed with a nested PCR assay.

Table 2. Comparison of the specificity of the newly developed biomarker PCR assays against fecal samples from within and outside the watershed.

EVALUATING BIOMARKERS AGANST FECAL SAMPLES- The 4 PCR primer sets were designed for specificity against a variety of nontarget fecal samples from within and outside the watershed (Table 2). Only the *Brevibacterium* clone biomarker did not amplify in any fecal sample with the exception of weak amplification (1 of 2 duplicates) by nested PCR in one duck and one goose sample from outside the watershed area.

Figure 1. Reconstructed phylogenetic tree of the *Brevibacterium* sp.



PHYLOGENTIC TREE- Reconstructed phylogentic tree of the *Brevibacterium* sp. based on 16S rRNA (Figure 1), which is closely related to *Brevibacterium avium*. Numbers at the nodes represent bootstrap values. The distance bar represents a 1% estimated sequence divergence.

QUANTIFYING POULTRY LITTER BIOMARKER IN ENVIRONMENTAL SAMPLES-A SYBR green qPCR protocol was developed and optimized using primers specific to the *Brevibacterium* sp. clone poultry litter biomarker. The standard curve of the qPCR assay for the biomarker is shown in Figure 2. Environmental samples from the area where soiled poultry litter was applied were tested for the presence of the biomarker using the qPCR assay (Table 3).

Figure 2. Standard curve of measured Ct values and standard deviations versus log plasmid biomarker concentration.

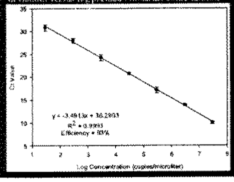


Table 3. Environmental samples tested for *Brevibacterium* clone poultry litter biomarker.

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

Major Conclusions

- Molecular methods successfully identified a novel biomarker unique to poultry litter found in a fecal-contaminated watershed relative to other sources of fecal pollution.
- A 16S rRNA-based real-time PCR assay targeting the poultry biomarker successfully amplified this target in environmental samples.
- Good correlation between the poultry litter biomarker and potentially pathogenic microbes *E. coli* and *Enterococcus* was observed in environmental water samples.

CORRELATION BETWEEN POULTRY LITTER BIOMARKER AND *E. COLI* AND *ENTEROCOCCUS*- The correlation between the poultry litter biomarker concentration (i.e., as quantified by qPCR) in water and litter samples and *E. coli* and *Enterococcus* as measured by most probable number counts is presented in Figures 3 and 4. In general, the *Enterococcus* MPN counts were well correlated (R^2 ranging from 0.75 to 0.89) with the concentration of the biomarker in the various samples.

Figure 3. Correlation between the concentrations of poultry litter biomarker, *E. coli* and *Enterococcus spp.* in litter samples.

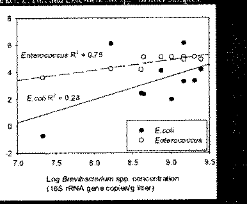
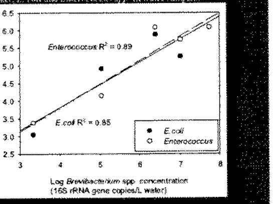


Figure 4. Correlation between the concentrations of poultry litter biomarker, *E. coli* and *Enterococcus spp.* in water samples.



Future Work

Future work will include the testing of environmental samples from within the watershed by the qPCR assay to evaluate the distribution of the poultry litter-specific biomarker as compared to indicator bacteria, heavy metals, and other water quality parameters. Additionally, testing of the poultry litter-specific biomarker against more fecal samples from other watersheds and additional avian fecal material will be conducted as the potential poultry litter biomarker was found in low abundance (i.e., a nested PCR approach was required for detection and only 1 duplicate was positive) in 2 non-target composite avian fecal samples collected outside the watershed. An independent lab is currently validating the results presented herein on a subset of samples.

Acknowledgements and References

This research was conducted in connection with work performed as retained experts in a pending legal case brought by the State of Oklahoma against several poultry integrators. Drs. Harwood and Olsen have been retained to serve as expert witnesses by the State of Oklahoma and have provided testimony regarding this research. This work was funded by the State of Oklahoma Attorney General's office to assess the extent of poultry waste contamination in the Illinois River Watershed.

The author's are grateful for the assistance provided by Kyle Collins, William Blackmare, James Jackson, Erin O'Leary Jepson and Michele Andrews. Additionally the authors acknowledge the Molecular Research Core Facility at Idaho State University for graciously allowing us the use of their laboratory space and equipment.

1. American Public Health Association. 2005. Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association, Inc., Washington, D.C.
2. Anderson, K., J. Whitlock, and V. Harwood. 2005. Persistence and Differential Survival of Fecal Indicator Bacteria in Subtropical Waters and Sediments. Applied and Environmental Microbiology 71:3041-3048.
3. Bernhard, A., and K. Field. 2000. Identification of Nonpoint Sources of Fecal Pollution in Coastal Waters by Using Host-Specific 16S Ribosomal DNA Genetic Markers from Fecal Anaerobes. Applied and Environmental Microbiology 66:1587-1594.
4. Desmarais, T., H. Solo-Gabriele, and C. Palmer. 2002. Influence of Soil on Fecal Indicator Organisms in a Tidally Influenced Subtropical Environment. Applied and Environmental Microbiology 68:1165-1172.
5. Edwards, U., T. Rogall, H. Blocker, M. Emde, and E. Bottger. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Research 17:7843-7853.
6. Lane, D. 1991. 16S/23S rRNA sequencing. In E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid sequencing techniques in bacterial systematics. John Wiley and Sons, New York, N.Y.
7. Leclerc, H., D. Mossel, S. Edberg, and C. Struijk. 2001. Advances in the Bacteriology of the Coliform Group: Their Suitability as Markers of Microbial Water Safety. Annual Reviews in Microbiology 55:201-234.
8. Tsen, H., C. Lin, and W. Chi. 1998. Development and use of 16S rRNA gene targeted PCR primers for the identification of Escherichia coli cells in water. Journal of Applied Microbiology 85:554-560.
9. USEPA. 2005. Microbial source tracking guide document. EPA/600-R-05/064. U.S. Environmental Protection Agency.
10. Van Donsel, D., E. Geldreich, and N. Clarke. 1967. Seasonal Variations in Survival of Indicator Bacteria in Soil and their Contribution to Storm-water Pollution. Applied Microbiology 15:1362-1370.
11. Wade, T., R. Calderon, E. Sams, M. Beach, K. Brenner, A. Williams, and A. Dufour. 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environmental Health Perspectives 114:24-28.

Identification and Validation of a Novel Poultry Litter Biomarker for Tracking Fecal Pollution

T.W. Macbeth[1], J.L. Weidhaas[1], R.L. Olsen[2], K.S. Sorenson[2], and V.J. Harwood[3]
[1]North Wind Inc, Idaho Falls, ID, [2]CDM, Denver, CO, [3]Univ. South Florida, Tampa, FL

The objective of this research was to develop a molecular biomarker specific to poultry litter and useful for tracking fecal pollution in a watershed affected by large-scale poultry farms. The 16S rRNA gene was targeted and community profiling conducted using terminal restriction fragment length polymorphism and clone library analysis to determine predominant populations in poultry litter that were conserved in soils to which litter had been applied. After screening numerous DNA sequences, a sequence with 97% similarity to previously isolated *Brevibacterium* sp. was selected for detailed evaluation. Polymerase chain reaction (PCR) primers specific to the *Brevibacterium* sp. were developed and tested against the original soil and litter samples, against closely related organisms identified in a BLAST database search, and against fecal samples from 32 other sources within and outside the watershed including beef cattle, dairy cattle, duck, goose, swine and human. The PCR primers amplified *Brevibacterium* sp. in all of the original soil and litter samples, and did not amplify DNA from a closely related *Brevibacteria spp.* identified in a BLAST search [DQ337537, isolated from swine lagoon effluent], or other fecal sources, except weakly in one goose and one duck sample that originated from outside the watershed.

Following validation of specificity of the *Brevibacterium* sp. biomarker, quantitative PCR (qPCR) with SYBR Green chemistry was developed. Environmental samples have been collected within and outside the affected watershed for analysis including, poultry litter, soil, runoff from the fields to which litter was applied, and river and lake waters. Analysis of these samples is ongoing. This research successfully identified a novel biomarker for poultry litter that is highly specific relative to other fecal sources within the watershed, and will allow a quantitative assessment of the distribution of the biomarker in environmental waters as a host-specific indicator of fecal pollution. This research was funded by the State of Oklahoma in on-going litigation against poultry integrators.

EXHIBIT C

IN THE UNITED STATES DISTRICT COURT FOR THE

NORTHERN DISTRICT OF OKLAHOMA

```
W. A. DREW EDMONDSON, in his )
capacity as ATTORNEY GENERAL )
OF THE STATE OF OKLAHOMA and )
OKLAHOMA SECRETARY OF THE    )
ENVIRONMENT C. MILES TOLBERT,)
in his capacity as the       )
TRUSTEE FOR NATURAL RESOURCES)
FOR THE STATE OF OKLAHOMA,   )
                             )
            Plaintiff,       )
                             )
vs.                          )4:05-CV-00329-TCK-SAJ
                             )
TYSON FOODS, INC., et al,    )
                             )
            Defendants.      )
```

- -

THE VIDEOTAPED DEPOSITION OF

VALERIE HARDWOOD, PhD, produced as a witness on behalf of the Defendants in the above styled and numbered cause, taken on the 18th day of July, 2008, in the City of Tulsa, County of Tulsa, State of Oklahoma, before me, Lisa A. Steinmeyer, a Certified Shorthand Reporter, duly certified under and by virtue of the laws of the State of Oklahoma.

A No.

Q Salmonella?

A No.

Q Any other bacteria?

A No. 09:13AM

Q Have you undertaken yourself to quantify fecal production levels by any animal in the IRW?

A No, I have not.

Q Have you undertaken quantification of bacteria loading from any particular source in the IRW? 09:13AM

A I have not.

Q Now, you submitted a journal article to the Journal of Applied and Environmental Microbiology; correct?

A That's correct. 09:14AM

Q And we were provided a copy of that a couple of days ago. You're on the editorial board of that journal?

A That's correct.

Q Okay. Have you discussed your article with any of your colleagues on that board? 09:14AM

A No, I have not. That wouldn't be -- you don't do that.

Q Okay. You submitted it on June 11, at least according to the cover E-mail; is that correct? 09:14AM

A Correct, uh-huh.

Q What is its status?

A It is pending -- it's in review, so that means that the folks who have received it to review, who are anonymous, are still reviewing it. 09:14AM

Q An article is reviewed before it's accepted?

A Correct, usually by two to three members of the editorial board and/or ad hoc reviewers who are not part of the editorial board.

Q Okay. Do you have any expectation as to when 09:14AM it might be accepted?

A Usually it's about two months, so I would think in August we will know something.

Q When you submitted the article, did you recommend peer reviewers? 09:15AM

A Yes. That's a common practice.

Q Who did you recommend?

A I don't remember. I'd have to look back.

Q Okay. Could you provide us with that information? 09:15AM

A Yes, I could, I think.

Q And you do not know who is reviewing your work; is that correct?

A No. It's anonymous.

MR. PAGE: Mr. Todd, I think it would be 09:15AM growing under certain conditions and the other group was growing under other responses and those responses were or those conditions were occurring at different times, then you could get difference in growth patterns. 09:18AM

Q Okay.

A However, I do need to qualify that by saying that the evidence for Enterococcus and E. coli growth in the environment is for very slow growth, so we're not talking about increasing by orders of 09:19AM magnitude in the sediment.

Q Okay. Flip to I think it's the next page of your packet. It's Table 4 of your submitted report, and if you look in the second column, which is numbers of samples tested, you report in your 09:19AM article testing ten litter sample, ten soil samples, ten edge of field samples, ten river water samples and six groundwater samples?

A Correct.

Q Why did you limit the number of river water 09:19AM samples to ten instead of including all of the tests that the State has done?

A Well, keep in mind that this article was written I believe, and I'd have to refresh my memory, but I believe it was written about a year 09:19AM ago, and so the strategy or the idea was that we used the samples that we had analyzed in the first round of PCR sampling because we had -- if you remember, we had several different groups of samples that were submitted for analysis, and so this 09:20AM was our first pass, and so we wrote the paper then based on this first pass of samples, and then are planning to do a follow-up later on with the remainder of the samples.

Q Okay. So when you say it was written a year 09:20AM ago, are you telling me that you were not editing until several months ago?

A Oh, yes, we were definitely editing it several months ago but, again, so when you start with a body of works -- this is a coherent body of work here. 09:20AM This is what you do in science. You have a coherent body of work. You publish that, and then you move on to the next stage. So the other samples were -- are conceptually for purpose of the publication in the next -- 09:20AM

MR. ELROD: John Elrod.

A -- in the next phase, which would be the next paper that we would we write.

Q Let me hand you No. 3. Professor, I've handed you what's been marked as Exhibit 3. Do you 09:21AM

A Yes, uh-huh.

Q Now, what is the purpose of having another lab cross validate North Wind's work?

A The purpose of having another lab cross validate is to -- is to -- well, just that. In 09:36AM
science -- in science cross validation by other groups -- independent validation of test results is a major -- is a way that we test the reliability of the assay.

Q Now, the E-mail we were just looking at refers 09:36AM
to Mike Sadowsky?

A Uh-huh.

Q Is that who you retained to cross validate?

A Yes. Mike Sadowsky at University of Minnesota is working on this. 09:37AM

Q Okay. Who is Mike Sadowsky?

A Mike Sadowsky is a professor of microbiology at the University of Minnesota. He's one of the leading environmental microbiologists in the country. 09:37AM

Q When was he retained?

A I believe it was May 2008, May or June 2008.

Q Did you all work out your contracting issues?

A Yes.

Q Okay. Have you worked with him before? 09:37AM

A Yes, I have worked with Mike. I've worked with Mike mostly on -- I've not -- just to clarify, I haven't co-authored anything with him, but I have worked with him on a book and worked with him on various microbial search tracking and environmental 09:37AM microbiology panels, expert workshop panels and things like that.

Q Now, what exactly was he retained to do?

A Mike's laboratory is going to utilize the qPCR assay and cross test some of the same samples that 09:38AM North Wind tested.

Q They're not going to recreate the entire North Wind process?

A That's correct.

Q Now, did you -- I take it you spoke with him 09:38AM in person about this?

A That's correct.

Q And you explained your procedure to him?

A Actually -- well, I very briefly explained the procedure to him, and then the details of the 09:38AM procedure were -- are in the -- are in the standard operating procedure of North Wind that was sent to him.

Q Okay. Did you explain your results to him?

A He knows about the -- he knows we're using the 09:38AM poultry litter biomarker in the watershed, in the IRW watershed, and that we're using it as a tracer or a marker for poultry litter contamination. I didn't go into depth explaining what we found beyond the fact that the qPCR assay seems to work really 09:39AM well.

Q And is he familiar with the context of this lawsuit?

A I wouldn't say he's familiar with it. I'd say he's heard about -- he's heard very briefly about 09:39AM the lawsuit but certainly not any of the details.

Q But he knows he's been retained to validate something that's being used in a lawsuit?

A Correct.

Q What materials was he given? 09:39AM

A Wow. The standard operating procedure of North Wind for the qPCR, the -- a set of samples that are coded that have no reference to source, and a plasmin, so a piece of DNA that has the biomarker sequence cloned into it so he can use that for a 09:40AM positive control.

Q How many samples was he given?

A Somewhere around 30 I believe.

Q Do you know which samples he was given?

A I can't tell you off the top of my head. I 09:40AM

EXHIBIT D

AEM Applied and Environmental Microbiology

HOME HELP FEEDBACK SUBSCRIPTIONS ARCHIVE SEARCH

Applied and Environmental Microbiology

To contact an editor, click here.

Editor in Chief
L. Nicholas Ornston (2011)
Yale University

Minireview Editor
Daniel J. Arp (2009)
Oregon State University

Editors

Axel A. Brakhage (2011)
HKI and University of Jena

Daniel Cullen (2012)
USDA Forest Products Laboratory

Harold L. Drake (2012)
University of Bayreuth

Katharine J. Gibson (2011)
DuPont Experimental Station

Lone Gram (2011)
Technical University of Denmark

Mansel W. Griffiths (2010)
University of Guelph

Sophia Kathariou (2012)
North Carolina State University

Michael J. Larkin (2012)
The Queen's University of Belfast

Laura G. Leff (2011)
Kent State University

Frank E. Löffler (2013)
Georgia Institute of Technology

Charles R. Lovell (2010)
University of South Carolina

Editorial Board
Yousef Abu Kwaik (2011)
Luis A. Actis (2011)
Eric Altermann (2009)
Gary Andersen (2010)
Thomas Andlid (2010)
Adam Arkin (2009)
Beate Averhoff (2010)
Andrea Azcarate-Peril (2009)
Felix J. Baerlocher (2009)
Michael Bagdasarian (2009)
Bert Bago (2010)
Christopher E. Bagwell (2011)
Alan Bakalinsky (2009)
Katherine H. Baker (2009)
József Baranyi (2010)
Tamar Barkay (2011)
Florian Bauer (2009)
Gwyn A. Beattie (2011)
Harry Beller (2009)
Andrew K. Benson (2009)
Peter L. Bergquist (2009)
Tom Besser (2010)
Arvind A. Bhagwat (2009)
K. Johanna Bjorkroth (2010)
Rafael Blasco (2010)
Eckhard Boles (2009)
Paola Bonfante (2009)
Violaine Bonnefoy (2010)
Kathryn J. Boor (2010)
Katherine A. Borkovich (2009)
James Borneman (2011)
Dulal Borthakur (2009)
Thomas L. Bott (2009)
Kostas Bourtzis (2009)
Gesche Braker (2011)
Michael G. Bramucci (2011)
Byron Brehm-Stecher (2010)
Frederick Breidt, Jr. (2010)
Neil C. Bruce (2010)
Alison Buchan (2011)
George Bullerjahn (2010)
Mary Beth Leigh (2010)
Jeffrey T. LeJeune (2009)
Michael Lemke (2011)
Tine Rask Licht (2011)
Celeste Linde (2011)
Markus Linder (2011)
Nic D. Lindley (2010)
John T. Lisle (2009)
Shuang-Jiang Liu (2011)
Wen-Tso Liu (2010)
Jon Lloyd (2010)
G. T. Macfarlane (2011)
Sandra Macfarlane (2011)
Robert Mach (2009)
Walter F. Mahaffee (2011)
Antonio Maldonado (2011)
Mike Manefield (2011)
Helene Marquis (2009)
Terence L. Marsh (2011)
Andrew P. Martin (2011)
Maria Esperanza Martínez-Romero (2010)
Jan Martinussen (2011)
Juergen Marxsen (2011)
Harold D. May (2010)
J. Vaun McArthur (2011)
Katherine McMahon (2009)
Brian McSpadden-Gardener (2010)
Rick Meinersmann (2009)
Jianghong Meng (2009)
William Metcalf (2011)
Patricia D. Millner (2009)
Donald K. Milton (2009)
Kiwamu Minamisawa (2011)
William W. Mohn (2011)
Sylvain Moineau (2011)
Istvan Molnar (2011)
Melanie Mormile (2010)
Volker Müller (2010)
Peter Muriana (2011)
Yuji Nagata (2009)
István Nagy (2010)
Cindy H. Nakatsu (2010)

Ellen L. Neidle (2012)
University of Georgia

Douglas H. Ohlendorf (2011)
University of Minnesota

Matthew R. Parsek, Cover Editor (2009)
University of Washington

Donald W. Schaffner (2010)
Rutgers, The State University of New Jersey

Janet L. Schottel (2013)
University of Minnesota

Alfred M. Spormann (2011)
Stanford University

Marylynn V. Yates (2009)
University of California—Riverside

Jizhong Zhou (2013)
University of Oklahoma

Chairman, Publications Board
Thomas E. Shenk

Director, Journals
Barbara M. Goldman

Production Editor
Barbara S. Slinker

Assistant Production Editors
Ellie Ghatineh
Michael E. Lerman

William Burkhardt III (2010)
H. J. Busscher (2010)
Mark P. Buttner (2009)
Murulee Byappanahalli (2009)
Douglas R. Call (2010)
Terri A. Camesano (2009)
Russell W. Carlson (2010)
Michael J. Carter (2010)
Jinru Chen (2011)
Shicheng Chen (2011)
Wilfred Chen (2009)
Yi Chen (2011)
Michael L. Chikindas (2010)
Luca Cocolin (2010)
James Cole (2010)
Michael T. Collins (2010)
James M. Cregg (2009)
Patricia Cruz (2010)
Tom Curtis (2010)
Rolf Daniel (2009)
Steven L. Daniel (2011)
Michelle Danyluk (2010)
Atin R. Datta (2010)
Frank B. Dazzo (2010)
Angelo DePaola, Jr. (2009)
Ronald P. de Vries (2011)
Alan DiSpirito (2011)
Jan Dolfing (2010)
Daniel P. Dougherty (2009)
Irina S. Druzhinina (2011)
Doris d'Souza (2011)
Nicole Dubilier (2010)
Siobain Duffy (2010)
Nancy DuTeau (2011)
Richard Eaton (2010)
Leo Eberl (2011)
T. S. Edrington (2010)
Henry L. Ehrlich (2010)
Christopher Elkins (2011)
Marie Elliot (2011)
David Emerson (2009)
K.-D. Entian (2010)
Slava Epstein (2010)
Danilo Ercolini (2009)
David Faguy (2011)
Joseph O. Falkinham III (2010)
Brian Federici (2009)
Juan Ferré (2009)
Matthew W. Fields (2011)
Turlough Finan (2010)
Steven E. Finkel (2010)
Madilyn Fletcher (2011)

Diane G. Newell (2010)
Walter G. Niehaus, Jr. (2010)
Brendan A. Niemira (2009)
Hideaki Nojiri (2009)
Kenneth M. Noll (2011)
Norbert Nowotny (2009)
George-John Nychas (2009)
Mark R. O'Brian (2009)
Kevin P. O'Connell (2011)
Ronald S. Oremland (2009)
Mark Osborn (2010)
Joerg Overmann (2010)
Hans Paerl (2011)
Rebecca Ehrlich Parales (2011)
Jakob Pernthaler (2009)
M. Julia Pettinari (2011)
Gregg Pettis (2011)
Laurent Philippot (2011)
Flynn Picardal (2010)
Roger W. Pickup (2010)
James L. Pinckney (2011)
Anton F. Post (2010)
Rolf Prade (2009)
Jim I. Prosser (2011)
Cheryl L. Quinn (2009)
John P. Quinn (2009)
Jos M. Raaijmakers (2011)
Gerald R. Reeck (2010)
Bernd Rehm (2009)
Gregor Reid (2009)
Walter Reineke (2010)
Geoffrey D. Robson (2011)
Margie Romine (2011)
Neil Rowan (2011)
Edward G. Ruby (2010)
Michael J. Sadowsky (2010)
Daâd A. Saffarini (2011)
Badal C. Saha (2010)
Andrew D. Sails (2010)
Sima Sariaslani (2009)
Brian Sauders (2010)
Patrick D. Schloss (2009)
Andreas Schmid (2011)
Andreas Schramm (2010)
Bernhard Seiboth (2011)
Verena Seidl (2009)
Gordon Shephard (2010)
Simon D. Silver (2011)
Pascal Simonet (2009)
Mitch Singer (2011)
Randall S. Singer (2009)
Brajesh Singh (2010)
Kaarina Sivonen (2011)

Steven Foley (2011)
Christopher Francis (2010)
James A. Fraser (2009)
Herbert L. Fredrickson (2010)
Masao Fukuda (2009)
Rebecca Gast (2011)
Manuela Giovannetti (2009)
Mark Gomelsky (2011)
Heidi Goodrich-Blair (2009)
Lawrence Goodridge (2009)
Joerg Graf (2011)
Irene R. Grant (2010)
Leslie Gregg-Jolly (2011)
Jean Guard-Bouldin (2010)
Mary Lou Guerinot (2011)
Martin W. Hahn (2010)
Theo A. Hansen (2010)
Hauke Harms (2010)
Steven Harris (2011)
Valerie J. (Jody) Harwood (2009)
Terry C. Hazen (2010)
Qiang He (2010)
Zhili He (2010)
Brian Hedlund (2011)
Hermann Heipieper (2011)
Alfredo Herrera-Estrella (2011)
David Hibbett (2011)
Russell T. Hill (2011)
Dallas G. Hoover (2009)
David J. Hopper (2010)
Hor-Gil Hur (2009)
Fumio Inagaki (2010)
Douglas Inglis (2009)
Janet K. Jansson (2010)
Lee-Ann Jaykus (2009)
Tom Jeffries (2011)
Paul R. Jensen (2011)
Carlos A. Jerez (2011)
Mike Jetten (2010)
D. Barrie Johnson (2011)
Yves Jouanneau (2009)
Ari Jumpponen (2009)
Juan Luis Jurat-Fuentes (2009)
David Kaplan (2010)
Levente Karaffa (2009)
Boran Kartal (2011)
Charles W. Kaspar (2010)
James E. Keen (2010)
Martin Keller (2009)
John J. Kelly (2009)
Joan L. Slonczewski (2011)
Kornelia Smalla (2009)
Hauke Smidt (2011)
Patricia A. Sobecky (2010)
John Spear (2010)
Andrew Spiers (2010)
Dirk Springael (2009)
Eric Stabb (2010)
Lucas J. Stal (2009)
Alfons J. M. Stams (2010)
Robert J. Steffan (2010)
Alexander Steinbüchel (2011)
Craig Stephens (2009)
John Stolz (2010)
Wolfgang Streit (2009)
Marc Strous (2011)
Joseph Sturino (2010)
Marcelino Suzuki (2010)
Zuzana Sýkorová (2011)
Ken Takai (2010)
Ralph Tanner (2011)
Gerald W. Tannock (2011)
Ron Teather (2010)
Andreas Teske (2009)
Chris M. Thomas (2009)
Michael G. Thomas (2009)
Ian P. Thompson (2011)
Tim Tolker-Nielsen (2009)
Eva M. Top (2009)
Effie Tsakalidou (2010)
Masataka Tsuda (2009)
Jos Vanderleyden (2010)
Wouter van der Star (2011)
Paul van der Wielen (2011)
Kumar Venkitanarayanan (2011)
Alain Vertes (2010)
Anne K. Vidaver (2011)
Adrián A. Vojnov (2010)
Gerrit Voordouw (2011)
Carmen Wacher (2010)
Michael Wagner (2010)
Irene Wagner-Döbler (2011)
Suzanne Walker (2009)
Judy D. Wall (2009)
Peng G. Wang (2009)
Todd Ward (2010)
Bart Weimer (2010)
Elizabeth M. Wellington (2010)
Alan Welman (2009)
Marvin Whiteley (2010)
Martin Wiedmann (2011)
Steve Wilhelm (2009)
Rolf-M. Wittich (2011)

Robert M. Kelly (2011)
Angela Kent (2010)
Lee Kerkhof (2009)
Philip J. Kersten (2010)
Mary Jo Kirisits (2010)
Maia Kivisaar (2010)
Staffan Kjelleberg (2011)
Martin G. Klotz (2011)
Allan Konopka (2011)
Joel E. Kostka (2010)
George Kowalchuk (2009)
Lee Krumholz (2009)
Indira Kudva (2010)
Kirsten Kuesel (2010)
Timothy M. LaPara (2009)
Laurie LaPat-Polasko (2009)
Gisele LaPointe (2010)
Luis Larrondo (2011)
Jared Leadbetter (2010)
Yin-Won Lee (2010)
Mark J. Wolcott (2010)
K. Eric Wommack (2009)
Thomas K. Wood (2011)
Randy Worobo (2010)
Michael Wyman (2009)
Dong Xu (2011)
Jian Xu (2010)
Jagjit Yadav (2011)
Ching-Hong Yang (2011)
Suleyman Yildirim (2011)
Jill Zeilstra-Ryalls (2011)
Chuanlun Zhang (2009)
Qijing Zhang (2011)
Erwin G. Zoetendal (2009)
Angeles Zorreguieta (2010)

Applied and Environmental Microbiology, a publication of the American Society for Microbiology (ASM), 1752 N St., N.W., Washington, DC 20036-2904, is devoted to the advancement and dissemination of applied knowledge as well as ecological knowledge, both applied and fundamental, concerning microorganisms.

HOME HELP FEEDBACK SUBSCRIPTIONS ARCHIVE SEARCH

J. Bacteriol. Microbiol. Mol. Biol. Rev. Eukaryot. Cell All ASM Journals

Copyright © 2009 by the American Society for Microbiology. All rights reserved.

EXHIBIT E

IN THE UNITED STATES DISTRICT COURT FOR THE

NORTHERN DISTRICT OF OKLAHOMA

W. A. DREW EDMONDSON, in his)
capacity as ATTORNEY GENERAL)
OF THE STATE OF OKLAHOMA and)
OKLAHOMA SECRETARY OF THE)
ENVIRONMENT C. MILES TOLBERT,)
in his capacity as the)
TRUSTEE FOR NATURAL RESOURCES)
FOR THE STATE OF OKLAHOMA,)
)
Plaintiff,)
)
vs.)4:05-CV-00329-TCK-SAJ
)
TYSON FOODS, INC., et al,)
)
Defendants.)

- -

VOLUME II OF THE VIDEOTAPED

DEPOSITION OF ROGER OLSEN, PhD, produced as a witness on behalf of the Defendants in the above styled and numbered cause, taken on the 11th day of September, 2008, in the City of Tulsa, County of Tulsa, State of Oklahoma, before me, Lisa A. Steinmeyer, a Certified Shorthand Reporter, duly certified under and by virtue of the laws of the State of Oklahoma.

A I'd have to look that up.

Q Was this a peer-reviewed publication?

A No.

Q Dr. Olsen, have you ever authored a peer-reviewed publication describing the results of 08:40AM a principal component analysis and identifying a source of contamination based upon those results?

A No.

Q Are you familiar with the peer review process that occurs in connection with publication? 08:41AM

A It's different with every journal.

Q You understand the idea is to have scientific work reviewed by other competent scientists, who aren't personally involved in the project; as a general matter, you agree with that as a definition 08:41AM for peer review?

A Well, you've just stated it yourself. So depends on, you know, the journal and -- but that's overall the purpose of it.

Q Okay. With that working definition, Dr. 08:41AM Olsen, have you had your work, your principal component analysis and your interpretation of those results in terms of source peer reviewed in this case?

A For publication? 08:41AM

Q Peer reviewed by anyone who -- any scientist who is not retained by the plaintiffs in this case.

A Well, everything that we've done and all the reviews that we've had other people do besides myself and Dr. Chappell have been by people retained 08:42AM by the plaintiffs. So there's no other person, besides your experts, that have not been retained by the State of Oklahoma for this case.

Q Just to clear it up and make sure our Record is clear, Dr. Olsen, you have not had your principal 08:42AM component analysis peer reviewed by scientists outside of this litigation; is that right?

A That's correct.

Q You started on this line of questions when I was asking you about Rick Chappell. Other than 08:42AM physically running the Sysstat program, what other services or support did Dr. Chappell or Mr. Chappell provide?

A Well, we went over what sections he wrote.

Q Right. 08:43AM

A So you can kind of --

Q Let's set that aside.

A Well, you can see the things that he did. Like he created, with Drew Santini and my help, the final database that was used in the PCA. He helped 08:43AM

EXHIBIT F

IN THE UNITED STATES DISTRICT COURT
FOR THE NORTHERN DISTRICT OF OKLAHOMA

STATE OF OKLAHOMA, ex rel,
W. A. DREW EDMONDSON,
in his capacity as ATTORNEY GENERAL
OF THE STATE OF OKLAHOMA,
and OKLAHOMA SECRETARY
OF THE ENVIRONMENT
C. MILES TOLBERT, in his capacity as
the TRUSTEE FOR NATURAL RESOURCES
FOR THE STATE OF OKLAHOMA,

Plaintiffs,

Case No. 4:05-cv-00329-GKF-SAJ

vs.

TYSON FOODS, Inc.,
TYSON POULTRY, INC.,
TYSON CHICKEN, INC.,
COBB-VANTRESS, INC.,
AVIAGEN, INC.,
CAL-MAINE FOODS, INC.,
CAL-MAINE FARMS, INC., CARGILL, INC.,
CARGILL TURKEY PRODUCTION, LLC,
GEORGE'S, INC., GEORGE'S FARMS, INC.,
PETERSON FARMS, INC.,
SIMMONS FOODS, Inc.
WILLOWBROOK FOODS, INC.

Defendants.

EXPERT REPORT OF VALERIE J. HARWOOD, Ph.D.

55. *Nested Sybr green PCR.* When the PLB concentration was below detection limit in the QPCR assay, a nested variant of this assay (which is presence-absence, rather than quantitative) was used to determine if lower levels of the PLB were present. In this case DNA extracted from the environmental samples was first amplified by conventional PCR using universal bacterial (16S rRNA) primers. This primary amplification step was followed by a secondary amplification step with the PLB primers (the LA 35 set). The identity and purity of the PCR product was always checked by conducting a melting curve analysis. This nested Sybr green procedure allowed detection of the PLB in many samples in which the PLB was at too low a concentration to quantify. Of 40 total soil samples collected from fields that received land-applied poultry litter, 38 had detectable levels of the PLB. Of 187 water samples (including 3 reference unimpacted samples) 99 had PLB levels below the detection limit, but 88 water samples had detectable levels of the PLB, including 1 geoprobe (shallow groundwater) sample (GPGW-10-4-11-30-06). A total of 3 spring or groundwater samples had detectable or quantifiable concentrations of the PLB, demonstrating transport of poultry waste in the subsurface. Furthermore, two of the samples that contained quantifiable concentrations of the PLB (HFS16-BF2-03-8-27-05 and HFS22-BF2-01-8-1-06) were base flow samples, which consist mainly of groundwater. Figures 5 and 6 show the results of nested Sybr green PCR testing for the PLB in water and soil samples, respectively. Sites at which the PLB was detected, but was too low to quantify by QPCR are designated by black triangles.

VI. CONCLUSIONS

56. Testing of poultry litter, soils upon which poultry litter has been applied, and edge-of-field samples collected from ditches during runoff conditions all show high levels of fecal indicator bacteria, some of which approach the levels expected in raw sewage. When these bacteria reach the extensive network of IRW tributaries, they become dominant contributors to the fecal indicator bacteria loads that impair the use of the Illinois River and its tributaries as recreational waters. The fecal indicator bacteria concentrations observed in the IRW tributaries, including those that receive extensive recreational use, are not characteristic of those in rural areas that are unimpacted by fecal contamination; rather, they are similar to areas that are extensively impacted by sewage or large-scale animal farming. The pathogenic microorganisms that are excreted in poultry feces and land-applied on contaminated poultry litter can impact the health of those who use the river for recreation, and also penetrate into the groundwater and contaminate the area's rural drinking water source. Sampling of IRW surface

water, groundwater, soil and sediments has revealed a unique chemical and bacterial signature that indicates contamination by poultry; and this signature is not present in areas that are remote from poultry operations. The finding that a poultry litter-specific biomarker (PLB) is found in all environmental compartments tested in the IRW, from soil samples to edge-of-field samples to surface water and groundwater, firmly links a dominant portion of the indicator bacteria contamination to poultry waste, which is well known to contain important human pathogens such as *Salmonella* and *Campylobacter*. Thus, the disposal of poultry waste by land application in the IRW presents a substantial, serious and immediate threat to human health.

57. If land application of poultry litter continues in the IRW, the loading of bacteria and particulate matter, which contributes to water turbidity, will continue. Much of this particulate matter settles out in stream bottoms and forms a habitat where the microbial contaminants can survive for long time periods – on the order of months or longer. The quality of surface water and groundwater in the IRW will continue to decline and the threat to human health will remain or increase. If land application of poultry litter ceases a major source of microbial contamination to the IRW will be removed. Once land application ceases and rain events over a season scour the contaminated soils and sediments, microbial water quality should substantially improve and the threat to human health will substantially decrease.

58. My opinions in this matter are my own, and do not reflect an official view of the University of South Florida.

Valerie J Harwood

Valerie J. Harwood, Ph.D.
Associate Professor
Department of Biology
University of South Florida

EXHIBIT G

Identification and Validation of a Poultry Litter-Specific Biomarker and Development of a 16S rRNA Based Quantitative PCR Assay

Jennifer L. Weidhaas[1], Tamzen W. Macbeth[1], Roger L. Olsen[2], Valerie J. Harwood[3], *

1. North Wind, Inc. 1425 Higham Street, Idaho Falls, Idaho, 83402
2. CDM, 555 17th St., Suite 1100, Denver, CO 80202
3. * Department of Biology, University of South Florida, 4202 E. Fowler Ave., Tampa, Florida 33620, Phone: 813-974-1524, Fax: 813-974-3263 email vharwood@cas.usf.edu

Running title: Brevibacterium marker for fecal source tracking of poulty

ABSTRACT

A poultry litter-specific biomarker was developed for microbial source tracking (MST) in environmental waters. 16S rRNA sequences that were present in fecal-contaminated turkey and chicken litter were identified by terminal restriction fragment length polymorphism (T-RFLP). Cloning and sequencing of potential targets from pools of *E. coli, Bacteroides* or total bacterial DNA yielded four sequences that were ubiquitous in poultry litter and also contained unique sequences for development of target-specific PCR primers. Primer sensitivity and specificity were tested by nested PCR against ten composite poultry litter samples and fecal samples from beef and dairy cattle, swine, ducks, geese, and human sewage. The sequence with greatest sensitivity (100%) and specificity (93.5%) has 98% identity to *Brevibacterium avium,* and was detected in all litter samples. It was detected at low level in only one goose and one duck sample. A quantitative PCR assay was developed and tested on litter, soil and water samples. Litter concentrations were $2.2*10^7$ - $2.5*10^9$ gene copies/g. The biomarker was present in a majority of soil and water samples collected in and near areas where litter was spread, reaching concentrations of 2.9×10^5 gene copies$\cdot$g^{-1} in soil samples and 5.5×10^7 gene copies$\cdot$L^{-1} in runoff from the edges of fields. The biomarker will contribute to quantifying the impact of fecal contamination by land-applied poultry litter in this watershed. Furthermore, it has potential for determining fecal source allocations for total maximum daily load (TMDL) programs and ambient water quality assessment, and may be useful in other geographic regions.

INTRODUCTION

Excessive land application of poultry litter as a waste disposal mechanism has been linked to eutrophication of water bodies (28, 35, 39), the spread of pathogens (15, 19, 21), air and soil pollution with metals (11, 33) and groundwater contamination with nitrate (5). Despite these known effects, land application is still the typically practiced disposal method for poultry litter even though viable and economically favorable alternative disposal practices are available (7, 20).

Identification of the source of fecal pollution contaminating a watershed is of particular interest for protection of water resources and the safety of recreational waters. For example, TMDL assessments require identification of the source of contamination, which is also necessary for remediation of impaired waters(44). Current methods for detecting the presence of fecal pollution, which carries an increased risk of the presence of pathogenic microorganisms, involve the cultivation of fecal indicator organisms such as fecal coliforms in the family *Enterobacteriaceae* (Oklahoma Administrative Code, Title 785, Chapter 46). The U.S. EPA and many states recognize *Escherichia coli* and enterococci as indicators of freshwater recreational water quality (42).

Drawbacks to the use of indicator organisms which limit the ability of researchers to pinpoint sources of fecal contamination include the non-specificity of the fecal coliforms to one source (25, 43), variable survival rates of various indicator organisms (1) and the growth or extended persistence of these indicator organisms after release to the environment (12, 45). These drawbacks have lead to research into alternative methods for the assessment of human health risk

from microbial pathogens in recreational waters that do not include the culturing of fecal indicator organisms for identification and quantification of the source of fecal pollution (46).

A variety of microbial source tracking (MST) methods (for recent reviews see (17, 40, 47)) have been proposed as an alternative to cultivation of fecal coliforms. Some of these genotypic molecular based techniques have included library dependent methods (i.e., culture and isolate-based) such as ribotyping (10, 31) and repetitive element polymerase chain reaction (REP-PCR) (14). Library independent methods (i.e., detection of a genetic biomarker in extracted DNA) have also been developed using discovery techniques such as suspension arrays (8), subtractive hybridization (13, 26), and terminal restriction fragment length polymorphism (T-RFLP) (3), among others. Host marker specific targets have included *Enterococcus faecium* (37), *Bifidobacterium* and members of the *Bacteroidales* (3, 22, 38), among others. Relatively few microbial targets specific to poultry fecal material have been identified. To date *Enterococcus faecalis* (23), *E. coli* (10) and *Bacteriodes* (26) have been associated with poultry fecal material, but only the *Bacteroides* biomarker (26) was specifically associated with poultry and not other fecal sources The objective of this research was to identify a poultry litter-specific biomarker, validate its specificity against other sources of fecal material from within and outside the watershed and develop a 16S rRNA based real-time PCR assay for quantifying the biomarker in environmental samples. This work was carried out as part of ongoing litigation in which the plaintiff is the Oklahoma Attorney General.

METHODS

Sample collection. Litter samples were collected from ten separate facilities (poultry houses), nine chicken and one turkey facility. Litter samples were collected from 18 locations within each

poultry house through the entire depth of the litter. The subsamples (total volume of 4 to 5 gallons) from each house were composited, homogenized and split (riffle splitter) before placement into a sterile whirl pack (approximately 500 mL) and shipped on ice to the laboratory for analysis. Litter application areas in fields (soils) were sampled by collecting 20 subsamples on a predetermined grid pattern across a uniform subarea of one to ten acres in size. The zero to two inch sample from six inch soil cores were composited, disaggregated, sieved to 2 mm, ground, homogenized and split. Vegetation, feathers, and rocks were removed. The split soil samples (500 ml) were transported on ice to the laboratory. Nontarget fecal samples for specificity testing were collected as composites from groups of individuals (Table 3). Samples from beef cattle were collected from ten grazing fields, of which five were within the watershed and five were outside the watershed. Two independent duplicate samples were collected for each field, and each duplicate consisted of feces from ten scats. A total of 200 beef cattle scats were collected and composited into 20 samples. Duck and goose samples were collected in the same fashion, consisting of composites from ten individual scats, and independent duplicates were collected for each area. For ducks, three landing areas inside the watershed and two outside the watershed were sampled, while for geese, two landing areas inside and three landing areas outside the watershed were sampled. A total of 100 scats for duck and geese were collected and composited into 10 samples for duck and 10 samples for geese. Composite samples of fecal slurries were collected from swine facilities, one inside the watershed and one outside (2 duplicate samples) and dairy cattle facilities (one inside the watershed and two outside (2 duplicate samples each) human residential septic cleanout trucks (3 samples) and influent of three separate municipal wastewater treatment plants (3 samples). A total of 20 g of each fecal sample other than litter from each site was collected and was placed in a 20 ml, sterile,

polystyrene tube containing 10 ml of 20% glycerol and shipped on dry ice to the laboratory. All fecal samples were homogenized in the glycerol before DNA extraction. Discrete water samples from larger rivers and lakes were collected using a Van Dorn water sampler or with a churn splitter for discrete or composite samples. Samples from larger rivers were typically composites of 3 samples collected on a transect across the width of the river channel. Samples from smaller rivers were collected using automated samplers. Samples collected during high flow events were composited based on flow volume. Base flow samples were collected as grab samples. River samples were placed into sterile 1-L polystyrene bottles in duplicate and shipped on ice to the laboratory where they were filtered. Runoff samples from the litter application areas (e.g. edge of field runoff samples) were collected during or as soon as possible after rainfall events. Samples were collected either with a passive runoff collector for composite samples or with a dip sampler for discrete samples. Runoff samples were placed into sterile 1-L polystyrene bottles in duplicate and shipped on ice to the laboratory where they were filtered. Groundwater samples were collected directly from existing homeowner's wells or from hydraulically driven shallow probes. Spring samples were collected as grab samples or by using a peristaltic pump. All samples were placed into sterile 1-L polystyrene bottles and shipped onice to the laboratory where they were filtered.

Enumeration of Indicator Bacteria. Indicator bacteria (fecal coliforms, *E. coli* and enterococci) were enumerated according to standard methods using multiple tube fermentation (MTF) and calculation of the most probable number according to according to SM-9221F or SM-9230 (APHA, 2005). MTF tubes containing *E. coli* were identified using broth cultures supplemented with (MUG) (SM-9221F) (2).

Soil, Litter and Fecal Sample DNA Extraction. Genomic DNA was extracted from soil, liter and fecal samples with Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc.) following the manufacturer's instructions. Typically 0.25 g of soil or litter was used in each extraction. DNA was purified by size-exclusion chromatography. Sepharose CL-4B (Sigma-Aldrich) was resuspended in Tris-HCL and sterilized by autoclave at 121 °C for at least 20 minutes. Micro-bio spin columns (Bio-Rad Laboratories) were packed with 1 mL of Sepharose CL-4B through centrifugation. Sepharose columns were then washed twice with Tris-HCl buffer (pH 8) and 50 to 150 µl of sample was added. Purified DNA was concentrated with ethanol precipitation and re-eluted in 100 µL sterile water.

Water Sample DNA Extraction. Within 12 hours of receipt at the laboratory all water samples were filtered through a sterile Supor-200, 0.2 µM filter and frozen at -80°C. Filters were then shattered with sterile glass beads and vortexed vigorously for 15 minutes with sterile, DNase, and RNase free water to remove solids and cells from the filters. The cell suspension was removed from the centrifuge tubes by pipette and placed in a 2 mL bead beating tube from the Bio101 Fast®Spin® DNA extraction kits. The cells were centrifuged at 20,000 x g for 10 minutes, and the supernatant was decanted. Genomic DNA was then extracted using the Bio101 Fast®Spin® DNA extraction kits (QBiogene, Inc). The extracted DNA was quantified using a Nanodrop® UV-Vis Spectrophotometer.

T-RFLP Analysis. Extracted genomic DNA and/or cloned DNA was amplified with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM) labeled universal bacterial primers 8F-907R (16, 24), with *E.coli* genus specific primers (Tsen, et al. 1998), and *Bacteroidales* specific primers (Bernhard and Field, 2000). All PCR primers targeted the 16S rRNA gene. Triplicate PCR reactions were generated from each DNA extraction, combined and purified

using QIAquick PCR purification Kits (Qiagen). Approximately 200 ng each of PCR product was digested at 37°C for 6 hours with the *Msp*I restriction enzyme (20µ/µL) (New England BioLabs). Samples were denatured by heating to 95° C for 3 minutes followed by cooling to 4°C. The digested fragments were purified by ethanol precipitation.

Primer Design. Primers were designed using the ABI Primer Express v.2 program (Applied Biosystems, Foster City, CA) and were targeted to variable regions between the potential biomarker sequences and sequences of the top 20 closest related organisms in the GenBank database. The BLAST search (Basic Alignment Search Tool, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was used to check the specificity of each primer.

PCR Assay Conditions. PCR was used to amplify approximately 900 bp of the 16S rRNA genes from *Bacteria* for clone library construction. Each 25 µL PCR reaction included 0.4 mg mL^{-1} molecular-grade bovine serum albumin (BSA) (Sigma Chemicals), 1X PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.5 µM of both the forward (8F) (16) and reverse (907R) (24) primer (Invitrogen), 1U Taq DNA polymerase (Promega), 0.2 mM dNTP (Invitrogen), 1 µL DNA template, and molecular-grade water (Promega). Amplification was performed on a PerkinElmer Model 9600 thermocycler using the following conditions: 94 °C for 5 minutes, 30 cycles of 94 °C (1 minute), 55 °C (45 seconds), and 72 °C (2 minute). A final extension at 72 °C for 7 minutes was performed and the PCR products were held at 4°C. Specificity of the PCR primers to the poultry litter biomarker was evaluated with nested PCR by first amplifying non-target fecal samples by universal bacterial primers 8F, 907R and then amplifying by the potential poultry litter biomarker PCR primers. The nested PCR master mix and thermocycler conditions were similar to the universal PCR with the following exceptions: 1) forward and reverse PCR

primers were specific to the potential poultry biomarker as shown in Table 2, 2) the annealing temperature was 60 °C. Amplification by nested PCR was evaluated by gel electrophoresis.

Clone Libraries. Clone libraries were constructed from the original genomic DNA extracted from the soil and litter samples and amplified with either universal bacterial primers 8F-907R (16, 24), targeting the 16S rRNA genes of *Bacteria* or the *E. coli* genus specific primers V1SF-V3AR (41). The TOPO ® Cloning Reaction methods from Invitrogen ™ were followed for clone library construction. Two clone libraries were constructed (targeting *Bacteria* and *E. coli*) from pooled DNA samples (i.e., 1 µl of genomic DNA extract from each sample was added to the PCR reaction for inclusion into the clones) based on the abundance of the various potential biomarkers as evidenced by the T-RFLP profiles.

qPCR Assay Conditions. Quantitative PCR (qPCR) was used to amplify 530 bp of the 16S rRNA gene from *Brevibacterium spp*. DNA samples were diluted to final concentrations of 3 ng/µL DNA. Each 25µL qPCR reaction included: 1X SYBR Green Master Mix (Roche), 0.5 µM of both the forward (LA35F) and reverse primer (LA35R) (Invitrogen), 5 % DMSO, 5 µL of diluted sample DNA, and molecular-grade water (Promega). Amplification was performed in triplicate on a Biorad Chromo4 thermocylcer using the following conditions: 50 °C for 2 minutes, 95 °C for 15 minutes, 45 cycles of 95 °C (30 seconds), 60 °C (30 seconds), and 72 °C (30 seconds) with a plate read. The 45 cycles was followed by a final extension at 50 °C for 5 minutes. Immediately following the final extension was a melting curve from 70 °C to 90 °C, by 0.1 degree increments, holding for 5 seconds with a plate read. DNA standards ranging from $6*10^{-15}$ to 10^{-21} ng/ul were prepared from serial dilutions of clone plasmid DNA containing the sequence of interest and used to develop the standard curve and method detection limit. Gene copy numbers were calculated from concentrations of positive control standards assuming 9.124

* 10^{14} bp/ul of DNA and one gene copy per genome. Detection limits for the qPCR assay were approximately 2000 plasmid copies in *E. coli*/L water and 7.3 *10^4 plasmid copies in *E. coli*/gram of soil. Nested qPCR was performed by first amplifying DNA with the universal bacterial 16S rRNA 8F (16) and 907R (24) primers. The production of PCR products was confirmed on a 1.5% agarose gel. The 16S rRNA PCR products were purified with the QIAquick PCR purification kit (QIAGEN) were subjected to qPCR as previously described using the LA35F and LA35R primers for the poultry litter biomarker.

Phylogeny. The phylogeny of the LA35 clone was investigated using the following methods. The clone sequences were assembled and aligned with BioEdit v. 7.0.5.3 and sequences were checked for chimeras with the Ribosomal Database Project II Chimera Check program and Bellerophon. The 16S rRNA sequences of the closest neighbors to the clone sequences were downloaded for inclusion in the phylogenic analysis. Multiple sequence alignments were constructed with Clustal W alignment tool and manually aligned in BioEdit. The bootstraps (1000 resamplings), maximum likelihood and distance matrix analysis (Kimura), and the reconstruction of the phylogenetic trees (FITCH) were performed with the Phylip 3.65 package and in particular the programs SEQBOOT, DNAML, DNADIST, FITCH, CONSENSE, and RETREE. The reconstructed phylogenetic tree was visualized with PhyloDraw V. 0.8 (Graphics Application Lab, Pusan National University).

RESULTS

Identification of potential biomarkers by T-RFLP. A total of 20 T-RFLP profiles were generated from the 5 subsamples of each of the two litter and two soil samples. The T-RFs common among the subsamples and representing more than 1% of the community were selected

for cloning and sequencing (Table 1). A total of 3 *E. coli* T-RFs (i.e., T-RF 496.0, 498.9 and 500.8) and 3 *Bacteria* T-RFs (i.e., T-RF142.9, 147.3 and 158.9) were selected for cloning and sequencing. Clone libraries were constructed from PCR products amplified with *E. coli* specific primers (V1SF-V3AR) (41) or universal bacterial primers (8F-907R) (16, 24). A total of 300 plasmids from the clone libraries were randomly picked. T-RFLP analysis was carried out on each plasmid insert to identify which plasmids contained the T-RFs of potential biomarkers. Inserts containing the T-RFs of interest were sequenced and PCR primers were developed for those sequences containing mismatches as compared to BLAST database results of the top 20 closely related organisms. In all 4 PCR primers for members of 4 genera were developed; a *Brevibacterium spp.*, a *Rhodoplanes spp.*, a *Kineococcus spp.* and a *Pantoea ananatis* strain (Table 2). Two *E. coli* T-RFs were from plasmids that did not contain mismatches between the sequence of interest and the sequences of closely related organisms identified in a BLAST search and therefore were not appropriate biomarkers.

Evaluation of biomarkers against fecal samples. The PCR assays developed for the 4 potential biomarkers of poultry litter were tested for amplification against a variety of nontarget fecal samples from within and outside the watershed (Table 3). Only the *Brevibacterium* clone LA35 appeared to be a potential candidate biomarker for poultry litter in that did not amplify in any fecal samples with the exception of weak amplification in one duck and one goose sample from outside the watershed when analyzed with a nested PCR approach (i.e. PCR with universal bacterial primers and then with the *Brevibacterium* clone LA35 primers). The reconstructed phylogenetic tree of the *Brevibacterium* clone LA35 in relationship to other *Brevibacterium* spp. is presented in Figure 1.

Quantification of the poultry litter biomarker in environmental samples. A SYBR green qPCR protocol was developed and optimized using the LA35F and LA35R primers (Table 2) specific to the *Brevibacterium* clone LA35 poultry litter biomarker. The standard curve of the qPCR assay for the biomarker is presented in Figure 2. The detection limit of the qPCR assay was 6 gene copies/ul of extracted DNA.

Environmental samples from the potential poultry litter impacted watershed were tested for the presence of the biomarker with the qPCR assay (Table 4). A variety of samples from within the watershed were tested, some of which were expected to contain the biomarker (e.g., litter, contaminated soil, runoff samples), some of which had variable potential for higher biomarker levels (e.g., surface water), and some of which had lower potential for biomarker presence (i.e., groundwater samples).

The correlation between the poultry litter biomarker concentration (i.e., as quantified by qPCR) in water and litter samples and *E. coli* and *Enterococcus* as measured by most probable number is presented in Figures 3 and 4. In general the *Enterococcus* MPN counts were well correlated with the concentration of the biomarker in litter ($R^2 = 0.75$) and with the biomarker concentration in water samples ($R^2 = 0.89$). The correlation between *E. coli* concentrations and the biomarker in water samples was also strong ($R^2 = 0.85$) while *E. coli* was less tightly (but significantly) correlated with the biomarker in litter samples ($R^2 = 0.28$). Correlation of the biomarker with *E. coli* and *Enterococcous* spp. provides a line of evidence of the human health risk associated with the runoff from poultry litter application to fields although there is evidence that regrowth of these organisms is possible once they are introduced into the environment (36).

DISCUSSION

The *Brevibacterium* sp. poultry litter biomarker developed in this study was validated in terms of sensitivity (100%) against numerous positive (poultry litter) samples from different locations with the watershed and for specificity (93.5%) against composite non-target fecal samples. These practices are in accordance with recent critical reviews (34, 40) that strongly recommend MST method validation. Future efforts will attempt to extend the method validation outside the watershed and possible outside the region as this biomarker could be useful for identifying fecal pollution sources in other river systems and coastal waters.

The *Brevibacterium* clone LA35 poultry litter biomarker was most closely related to *Brevibacterium avium*, which is associated with bumble-foot lesions in poultry (32). *Brevibacterium* spp. were recently identified in spent mushroom compost that was originally derived from chicken litter and cereal straw (29). Additionally *Brevibacterium avium, Brevibacterium iodinum,* and *Brevibacterium epidermidis* were found to represent more than 7% of a 16S rRNA clone library originating from broiler chicken litter (27). Certain *Brevibacterium* spp. are associated with milk and cheese curds(6), human skin(9), and soils (30). *Brevibacterium* spp. have been associated with disease in humans although to date these opportunistic pathogens have only been isolated from immunocompromised patients (4, 9, 18).

As poultry litter is land-applied as a disposal practice (19, 33, 35), it was important to identify a marker that could survive the process of deposition on bedding and spreading on fields. Therefore, the T-RFLP screening process included both litter and contaminated soil samples. This strategy allowed for the rapid elimination of numerous targets that could be abundant in the poultry fecal material, but not as abundant in the litter and not present in the environment after

litter application. This strategy for marker identification is in contrast with the work by Lu and colleagues (2007) where a genome fragment enrichment method was used to identify microbial sequences specific to chicken feces. Based on the PCR assays developed from clone libraries of the genome fragments, 6 to 40% of the chicken fecal samples collected from a wide geographic region contained DNA that could be amplified by the various assays (26). In comparison the LA35 biomarker was found in all the poultry litter samples tested, although it should be noted that all of the samples were collected in the Oklahoma/Arkansas region.

The examination of environmental samples from within the poultry litter impacted watershed suggest a correlation between the application of poultry litter to a field and concentration of the biomarker in the receiving waters, as evidenced by the generally decreasing trend in biomarker concentration with decreasing concentration of fecal indicator organisms. These results indicate that the watershed is in fact being impacted by the application of poultry litter to fields within the watershed. However, the magnitude of the impact as measured by the distribution of the biomarker within the watershed cannot be quantified with the limited number of environmental samples processed to date. Future work will include the testing of environmental samples from within the watershed by the qPCR assay to evaluate the distribution of the poultry litter-specific biomarker as compared to indicator bacteria, antibiotics and heavy metals. Additionally, testing of the poultry litter-specific biomarker against more fecal samples from other watersheds and additional avian fecal material will be conducted as the LA35 poultry litter biomarker was found in low abundance (i.e., a nested PCR approach was required for detection) in two non-target composite avian fecal samples (i.e., a duck and a goose sample) from outside the watershed.

Conclusions

In summary a novel biomarker of poultry litter was identified and a 16S rRNA based real-time PCR assay was developed for this biomarker. The specificity of the assay (93.5%) was tested against 31 separate non-target fecal samples and sensitivity was tested against10 target litter samples (100%). The field applicability of the assay was evaluated by testing for the biomarker in environmental samples expected to have variable concentrations of the biomarker, which we hypothesized would be correlated with the concentration of fecal indicator bacteria. A generally positive correlation was found between biomarker concentration and fecal indicator bacteria concentration which was particularly strong for enterococci. The research presented herein is the first identification of a *Brevibacterium* spp. for microbial source tracking studies and is among the first quantifiable method for tracking of poultry fecal sources in environmental waters.

ACKNOWLEDGMENTS

This research was conducted in connection with work performed as retained experts in a pending legal case brought by the State of Oklahoma against several poultry integrators. Drs. Harwood & Olsen have been retained to serve as expert witnesses by the State of Oklahoma and have provided testimony regarding this research.

The authors are grateful for the assistance provided by Kyle Collins, William Blackmore, James Jackson, Erin O'Leary Jeapson and Michelle Andrews. Additionally the authors acknowledge the Molecular Research Core Facility at Idaho State University for graciously allowing us the use of their laboratory space and equipment.

References

1. **Anderson, K., J. Whitlock, and V. Harwood.** 2005. Persistence and Differential Survival of Fecal Indicator Bacteria in Subtropical Waters and Sediments. Applied and Environmental Microbiology **71:**3041-3048.
2. **APHA.** 2005. Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association, Inc., Washington, D.C.
3. **Bernhard, A., and K. Field.** 2000. Identification of Nonpoint Sources of Fecal Pollution in Coastal Waters by Using Host-Specific 16S Ribosomal DNA Genetic Markers from Fecal Anaerobes. Applied and Environmental Microbiology **66:**1587-1594.
4. **Beukinga, I., H. Rodriguez-Villalobos, A. Deplano, F. Jacobs, and M. Struelens.** 2005. Management of long-term catheter-related *Brevibacterium* bacteraemia. Clinical Microbiology and Infection **10:**465-467.
5. **Bitzer, C., and J. Sims.** 1988. Estimating the availability of nitrogen in poultry manure through laboratory and field studies. Journal of Environmental Quality **17:**47-54.
6. **Brennan, N., A. Ward, T. Beresford, P. Fox, M. Goodfellow, and T. Cogan.** 2002. Biodiversity of the bacterial flora on the surface of a smear cheese. Applied and Environmental Microbiology **68:**820-830.
7. **Bujozcek, G., J. Oleszkiewicz, R. Sparling, and S. Cenkowski.** 2000. High Solid Anaerobic Digestion of Chicken Manure. Journal of Agricultural Engineering Research **76:**51-60.
8. **Call, D., D. Satterwhite, and M. Soule.** 2007. Using DNA suspension arrays to identify library-independent markers for bacterial source tracking. Applied and Environmental Microbiology **41:**3740-3746.

9. **Cannon, J., F. Spadoni, S. Pesh-Iman, and S. Johnson.** 2005. Pericardial infection caused by *Brevibacterium casei.* Clinical Microbiology and Infection **11:**164-165.

10. **Carson, C., B. Shear, M. Ellersieck, and A. Asfaw.** 2001. Identification of Fecal *Escherichia coli* from Humans and Animals by Ribotyping. Applied and Environmental Microbiology **67:**1503-1507.

11. **Connor, R., M. Connor, K. Irgolic, J. Sabrsula, H. Gurleyuk, R. Brunette, C. Howard, J. Garcia, J. Brien, J. Brien, and J. Brien.** 2005. Transformations, Air Transport and Human Impact of Arsenic from Poultry Litter. Environmental Forensics **6:**83-89.

12. **Desmarais, T., H. Solo-Gabriele, and C. Palmer.** 2002. Influence of Soil on Fecal Indicator Organisms in a Tidally Influenced Subtropical Environment. Applied and Environmental Microbiology **68:**1165-1172.

13. **Dick, L. K., A. E. Bernhard, T. J. Brodeur, J. W. Santo Domingo, J. M. Simpson, S. P. Walters, and K. G. Field.** 2005. Host distributions of uncultivated fecal *Bacteroidales* bacteria reveal genetic markers for fecal source identification. Applied and Environmental Microbiology **71:**3184-3191.

14. **Dombek, P., L. Johnson, S. Zimmerley, and M. Sadowsky.** 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. Applied and Environmental Microbiology **66:**2572-2577.

15. **Edwards, D., and T. Daniel.** 1994. A comparison of runoff quality effects of organic and inorganic fertilizers applied to fescue grass plots. Journal of the American Water Resources Association **30:**35-41.

16. **Edwards, U., T. Rogall, H. Blocker, M. Emde, and E. Bottger.** 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Research **17:**7843-7853.

17. **Field, K., and M. Samadpour.** 2007. Fecal source tracking, the indicator paradigm, and managing water quality. Water Research **41:**3517-3538.

18. **Janda, W., P. Tipirneni, and R. Novak.** 2003. *Brevibacterium casei* Bacteremia and Line Sepsis in a Patient with AIDS. Journal of Infection **46:**61-64.

19. **Jenkins, M., D. Endale, H. Schomber, and R. Sharpe.** 2006. Fecal bacteria and sex hormones in soil and runoff from cropped watersheds amended with poultry litter. Science of the Total Environment **358:**164-177.

20. **Kelleher, B., J. Leahy, A. Henihan, T. O'Dwyer, D. Sutton, and M. Leahy.** 2002. Advances in poultry litter disposal technology – a review. Bioresource Technology **83:**27-36.

21. **Kelley, T., O. Pancorbo, W. Mercka, S. Thompson, M. Cabrera, and H. Barnhart.** 1994. Fate of Selected Bacterial Pathogens and Indicators in Fractionated Poultry Litter During Storage. Journal of Applied Poultry Research **3:**279-288.

22. **Kildare, B. J., C. M. Leutenegger, B. S. McSwain, D. G. Bambic, V. B. Rajal, and S. Wuertz.** 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. Water Research **41:**3701-3715.

23. **Kuntz, R., P. Hartel, J. Rodgers, and W. Segars.** 2004. Presence of *Enterococcus faecalis* in broiler litter and wild bird feces for bacterial source tracking. Water Research **38:**3551-3557.

24. **Lane, D.** 1991. 16S/23S rRNA sequencing. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid sequencing techniques in bacterial systematics. John Wiley and Sons, New York, N.Y.

25. **Leclerc, H., D. Mossel, S. Edberg, and C. Struijk.** 2001. Advances in the Bacteriology of the Coliform Group: Their Suitability as Markers of Microbial Water Safety. Annual Reviews in Microbiology **55:**201-234.

26. **Lu, J., J. Domingo, and O. Shanks.** 2007. Identification of a chicken-specific fecal microbial sequences using a metagenomic approach. Water Research **41:**3561-3574.

27. **Lu, J., S. Sanchez, C. Hofacre, J. Maurer, B. Harmon, and M. Lee.** 2003. Evaluation of Broiler Litter with Reference to the Microbial Composition as Assessed by Using 16S rRNA and Functional Gene Markers. Applied and Environmental Microbiology **69:**901-908.

28. **Mozaffari, M., and J. Sims.** 1994. Phosphorus availability and sorption in an Atlantic coastal plain watershed dominated by animal-based agriculture. Soil Science **157:**97-107.

29. **Ntougias, S., G. Zervakis, N. Kavroulakis, C. Ehaliotis, and K. Papadopoulou.** 2004. Bacterial Diversity in Spent Mushroom Compost Assessed by Amplified rDNA Restriction Analysis and Sequencing of Cultivated Isolates. Systematic and Applied Microbiology **27:**746-754.

30. **Onraedt, A., W. Soetaert, and E. Vandamme.** 2005. Industrial importance of the genus *Brevibacterium*. Biotechnology Letters **27:**527-533.

31. **Parveen, S., R. Murphree, L. Edmiston, C. Kaspar, and M. Tamplin.** 1999. Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human

and nonhuman sources of fecal pollution. Applied and Environmental Microbiology **65:**3142-3147.

32. **Pascual, C., and M. Collins.** 1999. *Brevibacterium avium* sp. nov., isolated from poultry. International Journal of Systematic Bacteriology **49:**1527-1530.

33. **Pirani, A., K. Brye, T. Daniel, B. Haggard, E. Gbur, and J. Mattice.** 2006. Soluble Metal Leaching from a Poultry Litter–Amended Udult under Pasture Vegetation. Vadose Zone Journal **5:**1017-1034.

34. **Santo Domingo, J., D. Bambic, T. Edge, and S. Wuertz.** 2007. Quo vadis source tracking? Towards a strategic framework for environmental monitoring of fecal pollution. Water Research **41:**3539-3552.

35. **Schroeder, P., D. Radcliffer, and M. Cabrera.** 2004. Rainfall Timing and Poultry Litter Application Rate Effects on Phosphorus Loss in Surface Runoff. Journal of Environmental Quality **33:**2201-2209.

36. **Scott, T., J. Rose, T. Jenkins, S. Farrah, and J. Lukasik.** 2002. Microbial Source Tracking: Current Methodology and Future Directions. Applied and Environmental Microbiology **68:**5796-5803.

37. **Scott, T. M., T. M. Jenkins, J. Lukasik, and J. B. Rose.** 2005. Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. Environmental Science and Technology **39:**283-287.

38. **Seurinck, S., T. Defoirdt, W. Verstraete, and S. D. Siciliano.** 2005. Detection and quantification of the human-specific HF183 *Bacteroides* 16S rRNA genetic marker with real-time PCR for assessment of human faecal pollution in freshwater. Environmental Microbiology **7:**249-259.

39. **Sharpley, A., T. Daniel, J. Sims, and D. Pote.** 1996. Determining environmentally sound soil phosphorus levels. Journal of Soil and Water Conservation **51:**160-168.

40. **Stoeckel, D., and V. Harwood.** 2007. Performance, design and analysis in microbial source tracking studies. Applied and Environmental Microbiology **73:**2405-2415.

41. **Tsen, H., C. Lin, and W. Chi.** 1998. Development and use of 16S rRNA gene targeted PCR primers for the identification of *Escherichia coli* cells in water. Journal of Applied Microbiology **85:**554-560.

42. **USEPA.** 2000. Improved enumeration methods for the recreational water quality indicators: enterococci and *Escherichia coli.* EPA-821/R-771 97/004. U.S. Environmental Protection Agency.

43. **USEPA.** 2005. Microbial source tracking guide document, EPA/600/R-05/064. U.S. Environmental Protection Agency.

44. **USEPA.** 2001. Protocol for developing pathogen TMDLs. EPA 841-R-00-002. U.S. Environmental Protection Agency.

45. **Van Donsel, D., E. Geldreich, and N. Clarke.** 1967. Seasonal Variations in Survival of Indicator Bacteria in Soil and their Contribution to Storm-water Pollution. Applied Microbiology **15:**1362-1370.

46. **Wade, T., R. Calderon, E. Sams, M. Beach, K. Brenner, A. Williams, and A. Dufour.** 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspectives **114:**24-28.

47. **Wuertz, S., and J. Field.** 2007. Emerging microbial and chemical source tracking techniques to identify origins of fecal contamination in waterways. Water Research **41:**3515-3516.

Table 1. Common T-RFs among replicates from two fecal-contaminated poulty litter samples and two soils to which the litter had been applied.

	Number of subsamples tested (number containing T-RF of interest)			
T-RF	Litter A	Litter B	Soil A	Soil B
	E.coli PCR products, digested with *MspI*			
496.0	4 (4)	5 (4)	5 (3)	5 (5)
498.9	4 (4)	5 (5)	5 (4)	5 (5)
500.8	4 (4)	5 (5)	5 (5)	5 (5)
	Universal bacteria PCR products, digested with *MspI*			
80.1	4 (4)	5 (5)	5 (0)	3 (3)
130.9	4 (3)	5 (5)	5 (1)	3 (0)
142.9	4 (4)	5 (4)	5 (2)	3 (2)
147.3	4 (4)	5 (5)	5 (5)	3 (2)
158.9	4 (3)	5 (5)	5 (4)	3 (2)
165.0	4 (3)	5 (5)	5 (4)	3 (2)

*Underlined T-RFs correlate to those organisms for which PCR primers were developed

Table 2. Nucleotide sequences and targets of primers used in this study.

Primer	Target	Sequence (5'-3')	Position	Tm (°C)	T-RF
LA35F	*Brevibacterium*	ACCGGATACGACCATCTGC	166-184	57	147.3
LA35R	clone LA35	TCCCCAGTGTCAGTCACAGC	717-736	58	
SA19F	*Kineococcus*	TACGACTCACCTCGGCATC	163-181	56	158.9
SA19R	*spp.*	ACTCTAGTGTGCCCGTACCC	602-621	55	
SB37F	*Rhodoplanes*	AACGTGCCTTTTGGTTCG	143-160	56	142.9
SB37R	*spp.*	GCTCCTCAGTATCAAAGGCAG	616-626	55	
SA15F	*Pantoea*	CGATGTGGTTAATAACCGCAT	490-510	56	500.8
SA15R	*ananatis*	AAGCCTGCCAGTTTCAAATAC	668-688	55	

Table 3. Specificity of the poultry litter biomarker assay tested against fecal samples from within and outside the watershed.

Fecal sample (inside or outside watershed)	Number of samples tested (Number of samples containing potential biomarker)			
	Brevibacterium clone LA35	*Rhodoplanes* clone SB37	*Kineococcus* clone SA19	*Pantoea ananatis* clone SA15
Beef cattle (outside)	5 (0)	5 (2)	5 (1)	5 (0)
Beef cattle (inside)	5 (0)	5 (3)	5 (5)	5 (1)
Dairy cattle (outside)	2 (0)	2 (1)	2 (1)	2 (1)
Dairy cattle (inside)	1 (0)	1 (1)	1 (0)	1 (0)
Swine (outside)	1 (0)	1 (1)	1 (1)	1 (0)
Swine (inside)	1 (0)	1 (0)	1 (0)	1 (0)
Duck (outside)	2 (1)*	2 (2)	2 (2)	2 (2)
Duck (inside)	3 (0)	3 (1)	3 (1)	3 (2)
Goose (outside)	3 (1)*	3 (3)	3 (2)	3 (2)
Goose (inside)	2 (0)	2 (2)	2 (1)	2 (1)
Human sewage (outside)	2 (0)	2 (2)	2 (2)	2 (1)
Human sewage (inside)	4 (0)	4 (3)	4 (1)	4 (1)

* One duplicate amplified when analyzed with a nested PCR assay.

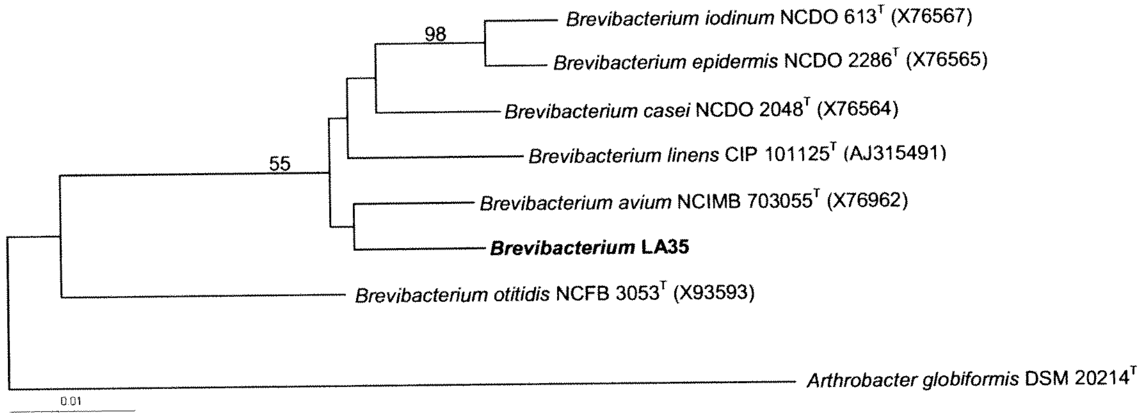

98
Brevibacterium iodinum NCDO 613T (X76567)
Brevibacterium epidermis NCDO 2286T (X76565)
Brevibacterium casei NCDO 2048T (X76564)
55
Brevibacterium linens CIP 101125T (AJ315491)
Brevibacterium avium NCIMB 703055T (X76962)
Brevibacterium LA35
Brevibacterium otitidis NCFB 3053T (X93593)
Arthrobacter globiformis DSM 20214T
0.01

Figure 1. Reconstructed phylogentic tree of the *Brevibacterium* spp. based on 16S rRNA. Numbers at the nodes represent bootstrap values (i.e. the number of times this organism was found in this position relative to other organisms in 1000 resamplings of the data). Bootstraps less than 50% are not shown. The closest cultured organisms as reported in an NCBI BLAST search are reported. The distance bar represents a 1% estimated sequence divergence.

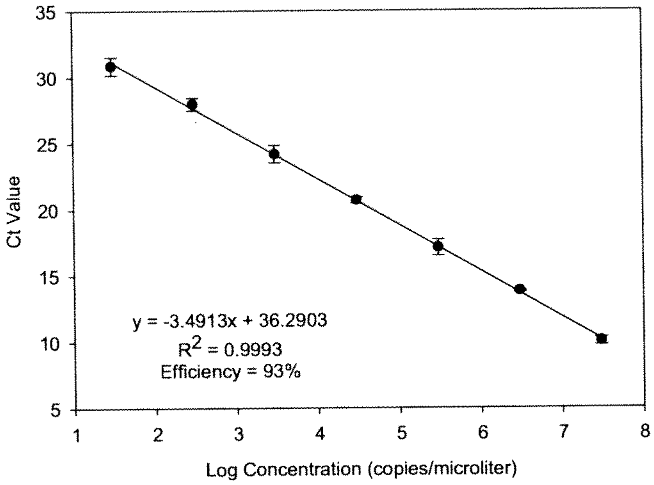


Figure 2. Standard curve of measured Ct values and standard deviations versus log plasmid biomarker concentration.

Table 4. Environmental samples tested for *Brevibacterium* clone LA35 poultry litter biomarker

Sample type	Number samples tested	% of samples containing biomarker [a]	% of samples quantifiable [b]	Range of biomarker present (16S rRNA copies/L water or g soil or g litter)
Litter	10	100	100	$2.2*10^7 \pm 7.1*10^6 - 2.5*10^9 \pm 9.5*10^7$
Soil	10	100	50	$7.0*10^3 \pm 4.4*10^2 - 2.9*10^5 \pm 2.0*10^4$
Edge of field runoff	10	100	100	$2.6*10^3 \pm 1.2*10^2 - 5.5*10^7 \pm 5.3*10^6$
River	10	50	20	$2.9*10^3 \pm 8.6*10^2 - 3.2*10^4 \pm 6.8*10^3$
Groundwater	6	0	0	Not applicable

[a] indicates the percent of samples in which the biomarker was identified by qPCR or nested qPCR methods

[b] indicates the percent of samples for which a quantifiable number of biomarker genes were measured by qPCR

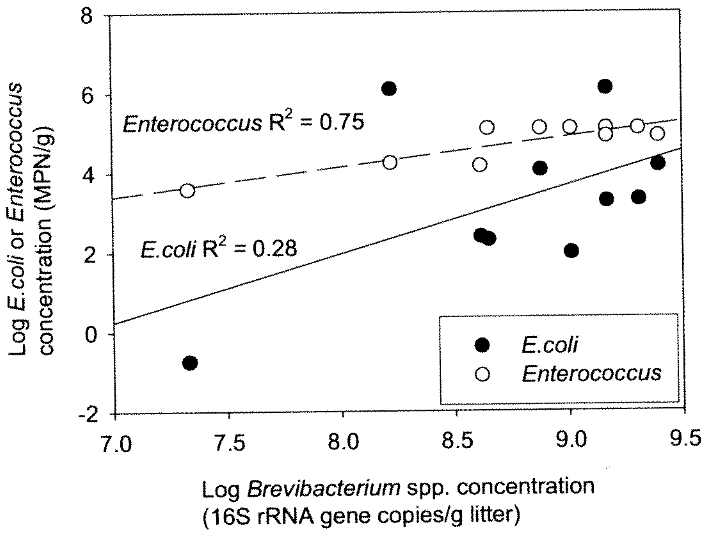


Figure 3. Correlation between the concentrations of poultry litter biomarker, *E. coli* and *Enterococcus spp.* in poulty litter samples.

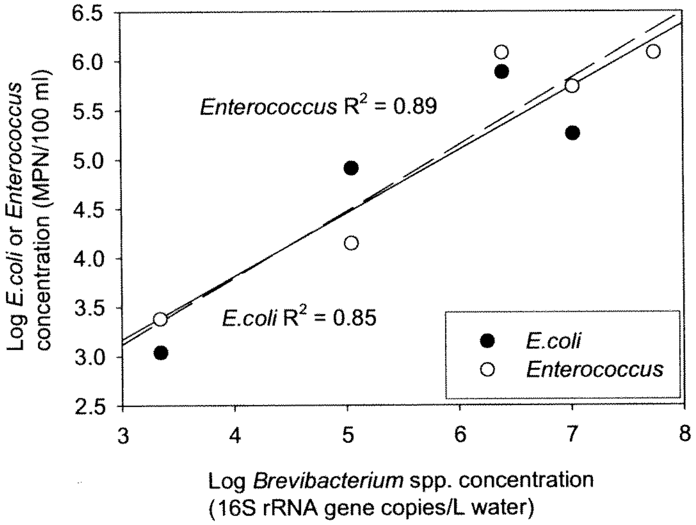


Figure 4. Correlation between the concentrations of poultry litter biomarker, *E. coli* and *Enterococcus* spp. in water samples.